DEVELOPMENT OF PROTOCOLS FOR AVOCADO TISSUE CULTURE: SOMATIC EMBRYOGENESIS, PROTOPLAST CULTURE, SHOOT PROLIFERATION AND PROTOPLAST FUSION

By

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by

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To my wife Nia,

for her tremendous sacrifice during these long years of study,

and to my sons, Lintang and Edgar,

for my not being there during their early days in this world.

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Avocado, *Persea americana* Mill., is an important fruit crop and is cultivated in tropical and subtropical regions. Despite its importance, commercial production has mostly depended on only a few rootstock and scion cultivars. Avocado improvement by conventional breeding has been slow due to a long juvenile period, low fruit set, genetic heterogeneity, lack of genetic information regarding horticultural traits and inefficiency of breeding techniques. Biotechnology, including somatic cell genetics and genetic transformation, has great potential for improving perennial fruit species, including avocado. The use of biotechnology for improving avocado is dependent on *in vitro* protocols, including efficient somatic embryogenesis, protoplast isolation and subsequent regeneration of plants from protoplasts, genetic transformation and shoot proliferation for propagating unique regenerants. This study was undertaken to develop the cell culture system for improving avocado.

Embryogenic cultures have been induced from several avocado genotypes and elite cultivars. Conditions for maintenance of embryogenic cultures have been determined, and efficient somatic embryo development from embryogenic cultures has been described. Protoplasts have been isolated from embryogenic cultures, and somatic embryo development from protoplast-derived cultures has been obtained. Although plant recovery from somatic embryos has been achieved, the efficiency of conversion, or germination has been low.

Interspecific protoplast fusion between embryogenic avocado cultures and leaf mesophyll protoplasts of *Nectandra coriacea* (Sw.) Griseb. , *P. borbonia* (L.) Spreng. and *P. pachypoda* has been attempted in an effort to produce interspecific somatic hybrids with resistance to phytophthora root rot caused by *Phytophthora cinnamomi* Rands; however, putative hybrid plants of the former and embryogenic cultures of the latter could not be confirmed by RAPD analysis. The somatic hybridization experiments were limited by the availability of protoplasts from the non-avocado parents. Whether or not protoplast fusion is a viable method for overcoming sexual incompatibility between avocado and its wild relatives remains unresolved.

The *in vitro* protocols for avocado that have been developed have significant implications for avocado improvement using biotechnology. A research collaboration has already demonstrated the feasibility of genetic transformation of embryogenic cultures of avocado.

CHAPTER 1
INTRODUCTION

Avocado, *Persea americana* Mill., is one of the most important fruit crops of the world. It has been consumed to some extent as a replacement for meat by native peoples of tropical America since antiquity (Popenoe, 1927). The avocado fruit has a high nutritional value and energy content and is a source of antioxidants, fruit protein and soluble fiber (Bergh, 1992 b). The high energy content of the avocado fruit is due to its high "good" fat content, which ranges from 3 to 30% fresh weight, depending on the cultivar. Avocado fat is 82% monounsaturated, of which 95% is oleic acid; 8% is polyunsaturated and 10% is saturated fat (Bergh, 1992b). Avocado oil is used as a cosmetic (Purseglove, 1968; Bergh, 1992a, b).

World avocado production in the last 5 years has averaged ca. 2 million MT (FAO, 1997). Among other fruits, world production of avocado ranks 10th after *Musa* (banana and plantain), citrus, grape, apple, mango, pear, plum, peach and papaya (FAO, 1992). In the USA alone, avocado's contribution to the economy during 1989-1992 was ca. $211 million annually. Among fruit crops, this is the sixth after citrus, grapes, apples, peaches and pears (Anonymous, 1992).

Despite its nutritional and economic importance, avocado genetics are not well understood. This is in large part due to typical problems of breeding perennial species, e.g., low fruit set, high heterogeneity and a long juvenility period. These difficulties have impeded conventional breeding for addressing problems faced by the avocado industry (Pliego-Alfaro & Bergh, 1992). Consequently, the avocado industry worldwide has mostly relied on a few scion cultivars such as 'Fuerte' and 'Hass' and a few rootstock cultivars such as 'Duke 7', 'Thomas', and 'Barr Duke', all of which originated from

chance seedlings (Bergh, 1976). The major breeding objectives for avocado involve development of better quality fruit and scion shoot, and rootstock (Bergh, 1975, 1976; Bergh & Lahav, 1996). Development of rootstocks that are tolerant of root rot disease caused by *Phytophthora cinnamomi* (PRR), for example, is urgently needed. Phytophthora root rot is the largest production problem around the world (Gustafson, 1976). In the USA alone, the avocado industry has been losing ca. 200 ha annually due to PRR. Over 60% of avocado groves in the USA are affected by PRR, and losses exceed $30 million each year (Coffey, 1987).

Biotechnology offers some novel approaches for generating variability for plant improvement. These have special relevance for perennial fruit species, because existing superior cultivars could be altered for one or more specific traits. Using tissue culture techniques, somaclonal variants with resistance to bacterial leaf spot caused by *Xanthomonas campestris* pv. Pruni (E.F. Sm.) Dows have been produced in peach (Hammerschlag, 1992). Genetic variability that would otherwise be unavailable using conventional breeding approaches can also be exploited by somatic hybridization involving distantly related and sexually incompatible species, e.g., citrus (Grosser & Gmitter, 1990; Gmitter et al., 1992). Tetraploid plants produced by somatic hybridization can be used as parents for triploid scion breeding (Grosser & Gmitter, 1990). Gene transfer by genetic recombination can also generate variability by bridging the sexual barriers between species, phyla and kingdoms. Genes from bacteria, e.g., the Bt gene for the endotoxin from *Bacillus thuringiensis* (Williams et al., 1992) and the VHp gene for hemoglobin from obligate aerobic, Gram negative *Vitreocilla* (Holmberg et al.,1997); from viruses, e.g., CP genes for the viral coat protein and viral nonstructural genes (Beachy et al., 1990); and from humans, e.g., 2-5 Aase (2-5 oligoadenylate synthetase) and RNase L (ribonuclease L) cDNA induced by interferon in human (Ogawa et al., 1996) can be transferred into and expressed in plants.

Cloning horticulturally important genes, i.e., ripening genes and transformation of plants with these genes in antisense (e.g., tomato) has marked the development of new approaches for addressing post-harvest problems. In fruit trees, stable integration and expression of foreign genes, *nos* and *npt* II genes, and their inheritance in Mendelian fashion have been demonstrated recently in apple (James et al., 1996). This breakthrough suggests that this technology might have direct application for single gene improvement of elite clones, e.g., for pest and disease resistance and for altered ripening for quality and shelf life improvement.

The potential for biotechnology can only be realized after development of efficient protocols for plant regeneration from cell and tissue cultures. With respect to avocado, only preliminary studies have been published, which were reviewed by Pliego-Alfaro & Bergh (1992). These studies were undertaken in order to develop protocols for avocado somatic cell genetics and included:

1. initiation and maintenance of embryogenic cultures in semisolid and liquid medium;
2. somatic embryo maturation and germination;
3. protoplast isolation, culture and regeneration from embryogenic cultures;
4. shoot proliferation from juvenile materials;
5. protoplast fusion between avocado and *Nectandra coriacea* and small-seeded *Persea* species.

CHAPTER 2
LITERATURE REVIEW

Avocado

Use and Importance

Avocado is a fruit that is usually consumed fresh as a salad, dessert or garnish. Unlike other fruits, the avocado does not provide refreshing satisfaction upon consumption, but it gives the fullness sensation of a staple food. Avocado pulp is smooth and nutty, neither sweet nor acid, and of a bland nature (Bergh & Lahav, 1996). Superior cultivars have a nutty or anise-like flavor.

Avocado has been an important food in Mexico and Central America since antiquity, where it takes the place of meat in the diet of Central Americans (Popenoe, 1927). Avocados are appetizing, nourishing, cheap and available throughout most of the year (Popenoe, 1927). According to Purseglove (1968), avocado is the most nutritious of all fruit. Analysis of the nutrient composition of avocado (Table 2-1) indicates that the fat content is very high and can be ca. 30%. Avocado fat is 82% monounsaturated, of which 95% is oleic acid, 8% is polyunsaturated and 10% is saturated fat (Bergh, 1992b), and it therefore shares some of the benefits of olive oil. Colquhoun (1990) demonstrated that inclusion of 15–20% avocado in low fat diets lowered blood cholesterol and preserved levels of high density lipoprotein (HDL), which is good for the heart, whereas a low fat diet without avocado lowered both cholesterol and HDL levels. Avocado also has a very high density nutritional value, high protein level, vitamins and potassium. The health benefits of avocado consumption have been discussed by Bergh (1992a; b).

Table 2-1. Nutrient composition of edible pulp of 'Fuerte' avocado per 100 g

Component	Amount
Water	74.0 g
Energy component	
Protein	2.2 g
Lipid	17.0 g
Carbohydrate	6.0 g
Fiber	1.5 g
Vitamins (mg or unless stated otherwise)	
A	290 I.U.
C	14.00 mg
Thiamine	0.11 mg
Riboflavin	0.20 mg
Niacin	1.60 mg
Mineral (mg)	
Calcium	10.0 mg
Phosphorus	42.0 mg
Iron	0.60 mg
Sodium	4.0 mg
Potassium	604.0 mg

Source: Scora and Wolstenholme (1998) (in press).

Taxonomy

Avocado, *Persea americana* (2n = 2x = 24), is a member of the Lauraceae, which is mostly comprised of tree species, except for the plant parasite *Cassytha filiformis*. Avocado is the only economically important food species in this family. Other economically important species are used as spices, e.g., *Cinnamomum zeylanicum* Blume and *C. cassia* (Nees) Nees and Eberm. Ex Blume; as medicinal plants, such as *C. camphora* (L.) J. Presl.; as timber, e. g., *Nectandra* Roland ex Rottb, *Ocotea* Aubl and *Phoebe*; and as ornamentals, i.e., *Persea indica* Spreng (Schroeder, 1995).

Kopp (1966) divided the genus *Persea* into 2 subgenera, *Persea* and *Eriodaphne*, based on the morphology of reproductive structures of herbarium materials. The genus *Persea* includes a small number of species characterized by large fruits, while the subgenus *Eriodaphne* consists of a large number of species, most of which have small fruits. This distinct demarcation of the two groups has been further confirmed by grafting and hybridization studies. The members within each subgenus are graft and sexually compatible with each other, but are incompatible with members of the other subgenus (Bergh & Ellstrand, 1986; Bergh & Lahav, 1996). The members of subgenus *Eriodaphne* are mostly resistant to *Phytophthora* root-rot, while members of subgenus *Persea* are susceptible (Zentmeyer, 1980)

The subgenus *Persea* includes the commercial avocado, which has a thick edible pulp, and other closely related species, which have thin pulp, e.g., *P. schiedeana* Nees, *P. primatogena* Williams and Molina, *P. parviflora* Williams and *P. zentmyeri* Scieber and Bergh. The commercial avocado has been long recognized to have three horticultural races that are adapted to three different climatic conditions, i.e., the Mexican race, which is adapted to high elevation in the tropics, the Guatemalan race which is adapted to medium elevations in the tropics, and the West Indian race which is adapted to low tropical elevations (Popenoe, 1941). Popenoe (1941) developed systematic keys to distinguish the three races and referred to the Mexican race as *Persea drymifolia,* the Guatemalan race as *P. guatemalensis* and the West Indian race as *P. americana.* The races also differ in many horticultural traits (Table 2-2).

The classification of the horticultural races of commercial avocado and other *Persea* species is still in dispute. Kopp (1966) recognized only 4 species, *P. schiedeana, P. steyernarkii, P. floccasa* and *P. americana*, the latter of which consisted of var. *drymifolia* (Mexican race), var. americana (West Indian race) and var *nubigena*; while the Guatemalan race was not discussed. William (1966; 1967), using morphological data and the fossil record, proposed that the commercial avocado consisted of two species, each

Table 2-2. Comparison of the three horticultural races of *Persea americana.*

Characteristic		Race		
		Mexican	Guatemalan	West Indian
General	Native Region	Mexican highlands	Guatemalan highlands	Tropical lowlands
	Climatic adaptation	subtropical	subtropical	tropical
	Cold tolerance	most	intermediate	least
	Salinity tolerance	least	intermediate	most
	Iron chlorosis tolerance	intermediate	least	most
	Alternate bearing	less	more	less
Form	Internode	longest	long	shortest
	Twig lenticels	pronounced	absent	absent
	Bark roughness	less	less	more
	Stem pubescence	more	less	less
Leaf	Size	smallest	large	largest
	Color	green	green	pale green
	Flush color	greenest	reddest	yellowish-green
	Anise	present (usually)	absent	absent
	Underside waxiness	more	less	less
Flower	Season	early	late	intermediate
	Bloom to maturity	5–7 months	10–18 months	6–8 months
	Perianth persistence	greater	less	less
Fruit Stem	Length	short	long	short
	Thickness	medium	thick	thin
	Shape	cylindrical	conical	nailhead
Fruit	Size	tiny-medium	small-large	medium-very large
	Shape	mostly elongated	mostly round	variable
Fruit Skin	Color	usually purple	black or green	pale green-maroon
	Surface	waxy coating	variable rough	shiny
	Thickness	very thin	thick	medium
	Stone cells	absent	present	slight
	Pliability	membranous	stiff	leathery
	Peeling	no	variable	yes
Seed	Size ratio	large	often small	large
	Coats	thin	usually thin	thick
	Tightness in cavity	often loose	tight	often loose
	Surface	smooth	smooth	rough
Pulp	Flavor	anise-like, spicy	often rich	sweet, mild
	Oil content	highest	high	low
	Distinct fibers	common	less common	intermediate
Cold storage	Tolerance	more	more	less

Source: Bergh & Lahav (1996), Bergh (1975)

with two varieties, the native species and its progenitor. The Mexican avocado, *P. americana* var. *drymifolia*, is the progenitor of the West Indian race, *P. americana* var. *americana*), while the Guatemalan avocado (*P. americana* var. *guatemalensis*) is the progenitor of *P. nubigena* var. *nubigena*.

The most recent classification has been suggested by Scora and Bergh (1990) based on new data, including isozymes, leaf terpenes, morphology, physiology, field observations and molecular markers as follows:

Persea americana Mill.
- var. *americana* Mill.
- var. *drymifolia* (Select. & Cham.) Blake
- var. *guatemalensis* Williams
- var. *nubigena* (Williams) Kopp
- var. *steyermarkii* Allen
- var. *floccasa* Mez

Persea zentmeyeri Scieber & Bergh "Aguacate de Montana"
"Aguacate de mico"
Persea parviflora Williams, "Aguacate cimarron"
Persea primatogena Williams & Molina 'Guaslipe"
"Aguacate de anis"
Persea schiedeana Nees

In this new classification, the horticultural races are given botanical varietal status since the difference between them is not far enough (based on isozymes) to give the races species standing but is too far to be considered forms (Bergh & Ellstrand, 1986). The three horticultural races were also as different from one to another as to other varieties (Scora & Bergh, 1990).

Origin, Domestication and Distribution

Origin

Avocado may have originated in the Chiapas-Guatemala-Honduras region where wild avocado is still found (Kopp, 1966); however, the three horticultural races of

avocado may have evolved in different climatic conditions in geographical isolation from one another (Storey et al., 1986). The Mexican race is thought to have originated in the highlands of south-central Mexico, since primitive forms of Mexican avocado are found in that area (Storey et al.,1986). The Guatemalan race was believed to have originated in the interior valley of the highlands of Guatemala, north of Guatemala City. The West Indian race did not originate in the West Indies, since there is no record of the avocado from early explorations of the West Indies (Storey et al., 1986). Instead, it may have developed in the Pacific lowlands of Central America (ca. 82°–92° west longitude [Storey et al., 1986]). William (1976; 1977) argued that the West Indian race evolved from the Mexican race and probably became adapted to a warmer climate in northern Central America. This argument was based on the morphological similarity of the two races and the archeological remains of avocado in Peru that date from ca. 1500 BC.

Domestication

Avocado has been domesticated in Mexico since time immemorial (Popenoe, 1927). The word avocado is derived from the Spanish word *ahuacate* or *aguacate*, which is a corruption of the Aztec ahuacatl which is still used in parts of Mexico. The word *pahua*, from the Aztec *pauatl* meaning fruit, is used with reference to the West Indian and Guatemalan races of avocado in certain parts of Mexico. The word *palta* (in Quechuan) is used with reference to avocado in western South America.

Archeological remains of avocado seeds as old as 7000 BC have been recovered from caves of the Tehuacán area in Mexico. Other avocado seeds with younger carbon dating ranging from 6600 BC, 4000 BC, 3200 BC, 500 BC, 300 BC to 300–1500 AD have also been recovered (Smith, 1966; 1969). Interestingly, seed size appears to have increased over time, indicating selection for larger fruit (Smith, 1966). Avocado seeds excavated from 2 sites in the Moche Valley (Peru) have been carbon dated at 2000–1500 BC and 1500–1200 BC (Williams, 1976).

Distribution

When the Spanish arrived in the Americas, the avocado was being cultivated from Mexico to northern Peru (Hodgson, 1950; Storey et al., 1986). The Spanish conquistadors brought the avocado to Venezuela, the West Indies, and the Canary Islands (Bergh & Lahav, 1996). Eventually, avocado was cultivated in all tropical and subtropical regions. The avocado reached Spain in 1600, and was established on the east coast of Africa in Ghana in 1750 (Smith et al., 1992). The avocado was introduced to Singapore ca. 1830 and to the Philippines ca. 1890 (Burkill, 1935). Avocado was brought from Mexico to Florida in 1833 and to California in 1848 (Gustafson, 1976).

Production

World avocado production in the last 5 years has averaged ca. 2 million MT (FAO, 1997) and ranks 10th after *Musa* (banana and plantain), citrus, grape, apple, mango, pear, plum, peach and papaya (FAO, 1992). The major production areas are Mexico, USA, Brazil, Dominican Republic and Indonesia, respectively (Table 2-3). Other countries that are not leading producers but which export significant amounts of avocado include Australia, South Africa and Israel.

Avocado Breeding and Advances

Avocado breeding objectives have been directed toward improvement of scion (fruit and tree) quality and rootstock quality as summarized in Table 2.4 (Bergh, 1975; Bergh, 1976; Bergh & Lahav, 1996).

According to Bergh & Lahav (1996), most of the morphological variability in avocado is multigenic and only one dwarfing character from *P. schiedeana* is controlled by a single gene. To combine desirable characters of different cultivars or to recover intermediate traits of two extreme phenotypes can be achieved efficiently by crossing the

Table 2-3. Average avocado production 1992-1996.

Region	Country	Production (tons)
North and Central America	Mexico	767,904
	U.S. A.	179,073
	El Salvador	40,600
	Costa Rica	23,480
	Guatemala	22,895
	Dominican Republic	156,000
	Haiti	45,000
	Cuba	8,100
South America	Brazil	108,037
	Venezuela	45,940
	Colombia	73,963
	Chile	53,400
	Ecuador	13,376
	Peru	54,559
Asia	Indonesia	96,926
	Israel	54,494
	Philippines	24,100
Africa	South Africa	40,953
	Cameroon	43,200
	Zaire	46,900
	Congo	24,500
	Madagascar	21,300
Europe	Spain	44,008
	Portugal	14,940
Australia	Australia	12,970
WORLD		2,080,088

Source: FAO (1997) adapted from Bergh & Lahav (1996)

Table 2-4. Avocado breeding objectives for special characters

Special Character	Special Character
Fruit Qualities	
Medium size (200–300 g)	Thick ovate shape
Uniformity	Pulp:
-Skin	-Proper softening
-Medium thickness	-Appetizing color
-Readily peelable	-Absence of fibers
-Insect, disease tolerance	-Pleasing flavor
-Free from blemishes	-Long shelf life
-Attractive color	-Slow oxidation
Long tree storage	-Chilling tolerance
Seed:	-High oil content
-Small	-High nutritional value
-Tight in its cavity	
Shoot Qualities	
Spreading habit	Tolerant of chlorosis
Easy to propagate	Tolerant of other stresses
Strong grower	Short fruit maturation period
Tolerant to pests and diseases	Precocious
Tolerant of wind	Regular bearing
Tolerant of cold	Wide adaptability
Tolerant of heat	Heavy bearer
Tolerant of salinity	
Rootstock Qualities	
Conducive to high quality fruit	Easily grafted
Conducive to healthy, productive trees	Tolerant to *Phytophthora* root-rot and other disease
Free from sunblotch viroid	
Dwarfing or semi-dwarfing	Tolerant of salinity
Genetically uniform	Tolerant of chlorosis
Hardy and vigorous	Tolerant of drought
Easily propagated	Tolerant of other adverse soil condition

Source: Bergh (1975)

cultivars (Bergh & Lahav, 1996). However, this conventional method has been challenged recently (Lavi et al., 1991; Lavi et al., 1993).

Genetic studies from populations of self- and cross-pollinated avocados with respect to anise scent, fruit density, flowering intensity, fruit weight, harvest duration,

inflorescence length, seed size, and softening time, had significant nonadditive variances (Lavi et al., 1991). This reflected the insignificantly low value of narrow-sense heritability but the significant value of broad sense heritability (Lavi et al., 1993). These results indicated that hybridization should be aimed at increasing the genetic variance in progenies by selecting parents that are not only of superior phenotype, but which also include 10–30% of parents with inferior performance (Lavi et al.,1993). More recently, genetic associations between DNA fingerprint fragments and loci controlling important traits in avocado, e.g., fruit color, have been reported (Mhameed et al., 1995). Selection of progenies having these traits, e.g., fruit skin color, may be carried out early in the seedling stage using markers associated with these traits.

Breeding scion cultivars

Avocado production mostly relies on a few cultivars. Scion cultivars are dominated by 'Hass' and 'Fuerte' which have been cultivated commercially for 40 years in subtropical regions. Both cultivars were chance seedlings of unknown parentage (Bergh, 1976; Bergh & Lahav, 1996). Hass has several commercial weakness, for example, it produce fruits with size variability, some proportion of which are too small to be marketable and this problem is aggravated with tree age (Bergh & Lahav, 1996). Several selections have been made to replace 'Hass' (Bergh & Lahav, 1996), e.g., 'Gwen', 'Jim', 'Reed' and 'Lamb'. Cultivation of these new cultivars is still limited due to consumer preference for 'Hass' (Bergh & Lahav, 1996). Several local selections have been made, including 'Ettinger' and 'Iriet' (Israel), 'Ardith' (USA) and 'Sarwill' (Australia) (Bergh & Lahav, 1996).

Breeding rootstock cultivars

The threat of root-rot disease, caused by *Phytophthora cinnamomi* Rands, to the avocado industry in California was recognized as early as the 1920s (Coffey, 1986). To address this problem, a breeding program was established at the University of California in

the early 1950s. *Persea* species and related genera collected from Mexico, Guatemala, Honduras and Nicaragua were tested for resistance to Phytophthora root-rot (PRR). The results indicated that most of the *Persea* species that are resistant to PRR belong to the subgenus *Eriodaphne*, while *Persea* species in subgenus *Persea* (including avocado) were susceptible (Table 2-5). The resistant species in subgenus *Eriodaphne*

Table 2-5. List of *Persea* and related genera and their resistance to *Phytophthora* root-rot.

Species	Origin of collection	Resistance
Persea subgenus Persea		
P. americana	Cultivars in California	low
P. americana	Honduras, Mexico, Guatemala, Nicaragua	low
P. flocassa Mez	Mexico	low
P. gigantea	Honduras	low
P. schiedeana	Mexico, Guatemala, UCLA clone	low
P. nubigena L. O. Willm (= *P. gigantea* L. O. Willm.; = *P americana* var. *nubigena* Kopp)	Guatemala, Honduras, Mexico, Nicaragua, El Salvador	generally low with some exceptions.
Persea subgenus Eriodaphne		
P. alba Nees & Mart	Brazil	high
P. borbonia (L.) K. Spreng.	southern United States	usually high, some variabilitye
P. caerulea (Ruiz & Pavon) Mez	Venezuela, Costa Rica	high
P. donnel-smithii Mez.	Guatemala, Honduras	moderately high, variable
P. haenkeana Mez. (= *P. durifolia* Mez.	Peru	moderate
P. indica (L.) K. Spreng	Canary Islands	very low
P. lingue (Ruiz & Pavon) Nees	Chile	low
P. pachypoda	Mexico	high
P. longipes	Mexico	low
P. liebmani	Mexico	high
P. cinerascens	Mexico	high
P. skutchii	Honduras, Guatemala	high
Various species of *Nectandra*, *Ocotea* and *Phoebe*	Latin America	usually moderately high

Source: Modified from Zentmeyer (1980); Zentmeyer & Schroeder (1953/1954); Zentmeyer & Schroeder (1955); Zentmeyer & Thorn (1956)

were graft incompatible with avocado and other species of the subgenus *Persea*, but were graft compatible with members of their own subgenus (Frolich et al., 1958). The same relation hold true for sexual hybridization (Bringhurst, 1954; Bergh & Lahav, 1996).

The program at UC, Riverside produced the PRR-tolerant 'Duke 7' rootstock, which was a seedling of 'Duke' (Zentmeyer & Thorn, 1956; Zentmeyer et al., 1963). 'Duke 7' has become the most important rootstock in California, and is propagated clonally using the etiolation technique (Platt, 1976). Other selections-that are promising include 'Barr Duke' (a seedling of 'Duke 6'), 'D9' that induces dwarfing (from an irradiated 'Duke' parent scions), 'Thomas' (a survivor from a root-rot affected area), 'Martin Grande' (a hybrid of avocado and *Persea schiedeana*)-are all still in trial (Bergh & Lahav, 1996). The level of tolerance to PRR of those selections are better than the earlierly used rootstock 'Topa Topa' but not as high as the PRR-resistant *Persea* species in the subgenus *Eriodaphne*.

The absence of complete PRR resistance in the subgenus *Persea*, together with lack of information regarding the genetics of PRR-tolerance, has made breeding for PRR tolerant/resistant rootstock difficult.

Biotechnology and Its Potential for Avocado Improvement

Despite its importance, breeding avocado has been slow due to its long juvenile period, high genetic heterozygosity, low fruit set, lack of genetic information and inefficient breeding method (Lavi et al., 1991b; 1993). Systematic studies of the genetics (Lavi et al., 1991b) and development of molecular markers for some horticultural traits, including fruit skin color (Lavi et al., 1991a) may have a significant impact on breeding efficiency. Nevertheless, breeding root-rot resistant rootstocks would be very difficult if not impossible to achieve using conventional methods, due to the lack of a resistance gene

pool. Biotechnology, involving somatic cell genetics and gene transfer, may have an important role in widening genetic variability.

Genetic transformation with antifungal genes such as glucanase has been proposed as an alternative to combat disease (Lamb et al., 1992) and might be a viable way to develop a root-rot resistant rootstock. Somatic hybridization via protoplast fusion has been used to overcome sexual barriers in citrus (Grosser & Gmitter, 1990). Protoplast fusion technology could be an alternative way of combining the root-rot resistance traits of *Persea* species that are sexually and graft incompatible with avocado (Pliego-Alfaro & Bergh, 1992; Bergh & Lahav, 1996). Somatic embryogenesis and plant regeneration from nucellar explants have been reported for woody tropical and subtropical fruit species, e.g., citrus (Rangan & Murashige, 1969) and mango (Litz et al., 1982; Litz et al., 1995). This approach may have direct application for cheap clonal propagation of a PRR tolerant rootstock.

Avocado tissue culture has not been developed in comparison with other tropical tree fruit species, i.e., citrus or mango. Avocado tissue culture has been considered as either being in its infancy (Pliego-Alfaro & Bergh, 1992) or recalcitrant (Gardner, 1993), although tissue culture studies of avocado were initiated 50 years ago. To realize the potential of modern biotechnology for improvement of avocado, tissue culture protocols, including plant regeneration via somatic embryogenesis and from protoplasts, protoplast fusion and plant propagation through shoot proliferation, need to be developed.

Previous work on avocado tissue culture, including callus initiation, shoot culture, somatic embryogenesis and protoplast isolation are summarized in Table 2-6. Early reports were intended to study growth responses of fruit pericarp tissue *in vitro* (Schroeder, 1956; 1961; 1971). Subsequently, callus initiation was reported from various tissues, including flower parts, cotyledons, seedling stems and leaves (Table 2-6). Callus also was initiated from stems of other *Persea* species, including *P. nubigena, P. borbonia*

Table 2-6. Summary of *in vitro* studies of avocado, *Persea americana* Mill., and related species in the family of Lauraceae.

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Callus culture						
Schroeder, 1956	*P. americana* 'Fuerte'	post harvest physiology	fruit pericarp	not reported	not reported	callus (cell proliferation)
Desjardins, 1958	cv. was not reported	Sunblotch viroid replication study	stem	28 – 30°C irradiance was not reported	Liquid medium BM: inorganic salts similar to Gautheret (1942) and White (1954), glycine, nicotinic acid, pyridoxine, thiamin, calcium pantothenate, LCE, 2% sucrose PGR: NAA	callus at cut-end of the stem had cambium origin callus was white then turned brown
Schroeder, 1961	*P. americana* cv. was not reported	callus anatomy	pericarp	not reported	BM: Nitch, White PGR: 10 mg l^{-1} IAA	cell proliferation: tracheid-like and parenchymatous cells
Schroeder, 1971	*P. americana* 'Hass'	physical factor requirement	pericarp	light and temperature were tested	BM: Nitsch with FeEDTA in exchange for iron citrate PGR: 10 mg l^{-1} IAA	callus grew better under dark, optimum temperature was ca. 25°C
Blumenfeld & Gazit, 1971	*P. americana* 'Fuerte'	cytokinin requirement	cotyledon, mesocarp	27°C darkness RH = 80%	BM: Miller, 1963 PGR: as treatment	for cell proliferation, mesocarp required exogenous cytokinin while cotyledon did not.

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Schroeder, 1975	*P. americana* cv. was not reported	ex vivo floral behavior	floral parts	27±2°C dark	BM: Nitsch or MS with LCE PGR: Kin, IAA, NAA (concentration not specified)	extensive callus, rooted callus
Schroeder, 1977	*P. americana* cv. was not reported	callus longevity	cotyledon, stem, peduncle, petiole	25 – 27°C 90 – 150 foot candle	BM: Nitsch PGR: not specified	Callus proliferation still occured after 3 – 16 years.
Schroeder, 1978	*P. americana* cv. was not reported	UV irradiation	fruit pieces	25 – 27°C 90 foot candle	Nitsch medium	callus growth was affected by UV dose
Schroeder, 1979	*P. americana* cv. was not reported	callus longevity	2 cm stem segment with or without bud (etiolated and non etiolated seedlings)	25°C 100-400 foot candle 24 h	Nitsch as modified by Schroeder (1977)	massive callus formation at cut-end of the stem segment, shoot/ bud elongation
Young, 1983	*P. americana* 'Lula', 'Waldin'	propagation	leaf, from seedling	27°C 1800 lux, 16 h	BM: Anderson (1970) salts and vitamine, 1 casein hydrolysate, 30 g/l sucrose PGR: 1 mg l^{-1} BA, 1 mg l^{-1} 2,4 D	callus
Blickle et al., 1988	*P. americana*	sunblotch viroid study	seedling stems	not applicable	BM: MS, 7 g/l agar PGR: 1 mg l^{-1} IAA and 0.3 mg l^{-1} BA	callus

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Aaouine, 1986	*P. americana* cv. 'Topa Topa' *P. borbonia* *P. indica* *P. nubigena*	callus formation and proliferation	seedling stems	27°C, darkness	BM: ½ MS major salts (except $MgSO_4$), MS minor salts, 15 mg/l Suc, 30 mg/l i-inositol, 0.4 mg/l thiamine.HCl, 1 mg/l pyridoxine.HCl, 1 mg/l nicotinic acid, 2 g/l Gelrite. PGR: 0.3 mg/l 2,4-D, 1 mg/l 2iP	Callus proliferation with fresh weight increase of 7 fold of initial explant.
Kane et al., 1989	*P. palustris* (Raf.) Sarg.	organogenesis	2 cm^3 cotyledon pieces	4 weeks dark, 4 weeks light 25 ± 2° C	BM: MS PGR: 0.1 NAA, 10 2iP	profuse callus formation, but no organogenesis
Shoot cultures						
Schroeder, 1980	*P americana* cv. was not reported	propagation	same as Schroeder (1979)	25 -- 27°C 100 - 200 foot candle 24 h	BM: Nitsch and 4 mg l^{-1} caseine hydrolysate or MS PGR: 6 mg l^{-1} and 30 mg l^{-1} isopentyl adenine for Nitsch and MS BM respectively	masive callus at cut-end of stem segment, shoot/bud elongation
Pliego-Alfaro, 1981	*P. americana* 'Topa-Topa'	shoot proliferation	2–3 cm stem tips from *in vitro* elongated shoot from decapitated plumule-radicle axes	27°C, 40 µmol m^{-2} s^{-1}, 15 h.	BM (MS salts, 0.4 mg l^{-1} thiamineHCl, 100 mg l^{-1} i-inositol, 30 g/l sucrose, 8 g/l TC agar), 170 mg l^{-1} NaH_2PO_4. H_2O, 1 g/l neutralized, activated charcoal. PGR: 50 mg l^{-1} BA	Shoot elongation with frequent die back and leaf abscision
Young, 1983	*P. americana* 'Lula', 'Waldin'	organogenic study	bud	27°C 1800 lux, 16 h	BM: PGR: 0.2 mg l^{-1} BA, 0.2 mg l^{-1} IBA	poor elongation of bud

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Skene & Barlass, 1983	*P. americana* Open pollinated 'Fuerte'	Embryo rescue	immature embryos from abscised fruitlets (open and cont-rolled pollination)	27°/20°C, 50 µmol m^{-2} s^{-1}, 16 h. or darkness	BM: ½ MS liquid medium PGR: 0.5 mg l^{-1} BA	germination and multiple shoot formation only for embryos of 6 week-old or later
Skene & Barlass, 1983	*P. americana* Open pollinated 'Fuerte'	shoot proliferation	plumule-radicle axes of mature embryos	27°/20°C, 50 µmol m^{-2} s^{-1}, 16 h	10 ml liquid medium: BM: 2 PGR: 0.5 mg l^{-1} BA	multiple shoots, 4 – 5 shoots
Skene & Barlass, 1983	*P. americana* Open pollinated 'Fuerte'	shoot proliferation from resqued embryos	2 cm shoots from proliferating shoots	27°/20°C, 50 µmol m^{-2} s^{-1}, 16 h	Shoots were pretreated with 20 s dip in 1 N NaOH. BM: White's medium, pH = 9.0, Agar PGR: 1 mg l^{-1} IBA	30 – 50% rooting
Gonzales-Rosas & Salazar-Garcia,1984	*P americana* var. *americana R. antillana*	shoot proliferation	nodal and stem tips of 2 – 3 cm from seedlings	27 ± 1°C 500 lux 16 h	BM: MS PGR: 0.3 – 10 IBA and 0.01 – 3 Kin	90% cultures formed multiple shoots of 1.5 – 2 cm with scaly leaves
		rooting	nodal and stem tips of 2 – 3 cm from seedlings	27 ± 1°C 500 lux 16 h	BM: MS PGR: 7.5 – 10 IBA	50% stem segment developed root
Harty, 1985	*P. americana* 'Duke 7'	shoot proliferation	shoot apices with 4 – 6 leaf primordia from green-house grown seedlings	27°C irradiance - reported 16 h	BM: MS inorganic salts modified by Dixon & Fuller (1976), 1000 myo-inositol, 30 g/l sucrose, 0.5 mg l^{-1} pyridoxine HCl, 5 mg l^{-1} thiamine HCl, 5 mg l^{-1} nicotinic acid, 40 mg l^{-1} L-glutamine, 40 mg l^{-1} L-arginine PGR: 10 mg l^{-1} Kin	shoot multiplication

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Pliego-Alfaro et al., 1987	*P. americana* 'GA-13' 'IV-8'	shoot proliferation	nodal stem from heavily pruned trees ('IV-8') and grafted adult buds ('GA-13')	not reported	BM: Margara major salts (1984), MS minor, 0.4 mg l^{-1} thiamineHCl, 100 mg l^{-1} i-inositol, 30 g/l sucrose, 8 g/l TC agar PGR: 1 mg l^{-1} BA	shoot proliferation with declining rate due to shoot apical necrosis different growth requirement for different cultivar
		rooting assay	2 – 3 cm shoot tips from shoot cultures	not reported	two-step rooting method (Pliego-Alfaro, 1988)	30% of 'IV-8' 5% of 'GA-13'
Pliego-Alfaro & Murashige, 1987	*P. americana* 'Topa-Topa'	embryo germination	plumule-radicle axes	27°C, 32 µmol m^{-2} s^{-1}, 16 h	BM (MS salts, 0.4 mg l^{-1} thiamineHCl, 100 mg l^{-1} i-inositol, 30 g/l sucrose, 8 g/l TC agar), 1 g/l neutralized, activated charcoal.	shoot elongation from plumules
Pliego-Alfaro & Murashige, 1987		shoot proliferation	1–1.5 cm nodal stems developed from greenhouse-grafted adult buds	27°C, 32 µmol m^{-2} s^{-1}, 16 h.	BM, 170 mg l^{-1} NaH_2PO_4. H_2O, 1 g/l neutralized, activated charcoal. PGR: 50 mg l^{-1} BA	bud elongated to 0.5 – 1 cm stem (50% cultures)
Pliego-Alfaro, 1988	*P. americana* 'Topa-Topa'	rooting experiment	2–3 cm stem tips from *in vitro* elongated shoot from decapitated plumule-radicle axes	27°C, 32 µmol m^{-2} s^{-1}, 16 h.	Induction (3 days): BM with 0.3x MS salts PGR: 25 mg l^{-1} IBA. Development: BM, no PGR, 1 g/l activated charcoal	100% rooting in 4 – 8 weeks

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Pliego-Alfaro & Murashige, 1987	*P. americana* 'Topa-Topa'	rooting test of different phases of avocado shoots	2 – 3 cm stem tips from juvenile, adult and 4x grafted adult materials (rejuvenated)	27°C, 32 µmol $m^{-2} s^{-1}$, 16 h.	3 days in induction: BM: 0.3x MS salts PGR: 25 mg l^{-1} IBA. Development: BM, no PGR, 1 g/l activated charcoal	juvenile stems rooted 100%, adult stems rooted 0%, rejuvenated stem rooted 30%
Schall, 1987	*P. americana* 'Fuerte'	shoot proliferation	shoot tips and nodal stem from 2-4 yr. old tree grafted trees	27 ± 1°C light 16 h	BM (according to Nel et al.,1982): ½ MS salts, Morel (1948) vitamin, 100 myo-inositol, 30 g/l sucrose, 25 mg l^{-1} FeNa EDTA, 170 mg l^{-1} NaH_2PO_4. H_2O PGR: 1 – 2 BA	culture survival of 70%, multiple shoot formation with poor leaf quality
		rooting	shoot tips		BM, 0.5 mg/l IBA, 0.5 mg/l AC	77% of shoots rooted in 77 days and 80% of it survived upon acclimatizationl
Cooper, 1987	*P. americana* 'Fuerte'	shoot proliferation	shoot tips and nodal stem from seedling		BM: WPM PGR: 0.1 mg/l BA	multiplication rate of 3 shoots per bud
		rooting			BM, 3 g/l NAA	90-100% rooting
Vega-Solarzano, 1989	*P. americana* 'Colin V-33' and a West Indian race selection	propagation	etiolated buds/shoots (the phase of the shoots were not reported)	25°C 3000 lux 16 h	BM: ½ MS, 100 mg l^{-1} myo-inositol, 0.4 mg l^{-1} thiamin HCL, 30 g/l sucrose, 2 g/l Gelrite PGR: 2 mg l^{-1} BA, 2 mg l^{-1} GA (filter sterilized)	3 multiple shoots per explant

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Biasi et al., 1994.	*P. americana* 'Ouro-verde'	shoot proliferation	nodal stem from seedlings	not reported	BM: ½ MS Salts PGR: 3 mg l^{-1} BA	axillary bud growth
		rooting	shoot tips	not reported	according to Pliego-Alfaro (1988)	rooting frequency of 45%
Barringer et al., 1996	*P. americana* West Indian races:	shoot proliferation	embryonic axis	28°C 40 µmol m^{-2} s^{-1} 16 h.	BM: MS PGR: 13.2 µM BA, 0.05 µM NAA	formation of multiple shoots
	'Dade' 'Maxima', 'Tower 2'	shoot proliferation	*in vitro* shoots		BM: MS PGR: 4.4 µM BA, 0.05 µM NAA	multiplication rate of 3.2 per nodal segment in the 2nd subculture
	'Choquette'	rooting	*in vitro* shoots		BM: MS PGR: 4.9 or 9.8 µM IBA	30% rooting
Gonzales-Rosas et al., 1985	*P. schiedeana*	propagation	1 cm nodal stem segment and stem tips with 1 bud	27 ± 1°C 800 lux 16 h	BM: MS PGR: 1 – 3 IBA and 0.01 – 1 Kin	shoot elongation from the existing buds, extensive callus at the shoot base
		rooting			BM: MS PGR: 1 IBA, 3 Kin	rooted shoots and stem segment with extensive callus
Nel et al, 1982	*P. indica*	propagation: shoot proliferation	5 mm stem tips or 1 – 2 node stem from 1 year-old seedlings	24° C light 16 h	BM: ½ MS major, MS minor, 100 myo-inositol, 30 g/l sucrose, 25 mg l^{-1} FeNa EDTA, 170 mg l^{-1} NaH_2PO_4. H_2O, 80 mg l^{-1} adenine sulfate, 25 mg l^{-1} ascorbic acid, 2 glycine, 1 for each pyridoxine HCl, thiamine HCl, nicotinic acid, panthothenic acid. PGR: 2 mg l^{-1} BA	Shoot proliferation (12 shoot/node)

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Nel et al, 1982		propagation, rooting			BM, 2 mg l^{-1} IBA (liquid medium with filter paper bridge)	65% rooting after 7 weeks
Kane et al., 1989	*P. palustris*	Propagation, shoot proliferation	shoot tips from *in vitro* seedlings	15 ± 2° C 90 µmol m^{-2} s^{-1}, 16 h	BM: MS medium PGR: 0.25 mg l^{-1} BA,0.5 mg l^{-1} GA Liquid medium (10 ml), 100 rpm	shoot multiplication, 4 fold increase per 6 weeks
	P. palustris .	propagation, ex vitro rooting	3 cm cutting		dip for 5 min in 500 mg l^{-1} IBA, plug trays with Vegro Klay Mix A	100% rooting
Campos & Pais, 1996	*P. indica*	propagation, shoot proliferation	nodal stem from *in vitro* seedling	22 ± 2° C 11.5 W m^{-2} 16 h	BM: MS, 30 g/l sucrose, 8 g/l agar, pH = 7.0 PGR: 1 mg l^{-1} BA	shoot elongation from buds
		propagation, rooting	terminal shoot cutting 2 – 8 cm from shoot cultures		induction: 1–2 s dip in 1–5 mg/ml IBA development: BM with ½ MS medium, no PGR	100 % rooting
Wang & Hu, 1984	*Sassafras randaiense*	Propagation: shoot multiplication	1 cm shoot tips from 5 yr-old grafted trees	18 – 20°C, 2 klx cool white fluorescent light, 12 h	BM (MS salts with 2x FeEDTA and LS organic, 5% sucrose), 30% LCE, 100 mg l^{-1} malt extract, 50 mg l^{-1} glutamine, 50 mg l^{-1} arginine PGR: 5 mg l^{-1} Kin, mg l^{-1}, 0.05 NAA (60 mg l^{-1} Kin required for stage II)	shoot multiplication
		propagation: rooting	*in vitro* shoot ≥ 1 cm		BM PGR: 5 – 10 IBA liquid medium with filter paper support	rooting of ≤ 20%

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Nel et al, 1982		propagation, rooting			BM, 2 mg l^{-1} IBA (liquid medium with filter paper bridge)	65% rooting after 7 weeks
Kane et al., 1989	*P. palustris*	Propagation, shoot proliferation	shoot tips from *in vitro* seedlings	15 ± 2° C 90 µmol m^{-2} s^{-1}, 16 h	BM: MS medium PGR: 0.25 mg l^{-1} BA,0.5 mg l^{-1} GA Liquid medium (10 ml), 100 rpm	shoot multiplication, 4 fold increase per 6 weeks
	P. palustris .	propagation, ex vitro rooting	3 cm cutting		dip for 5 min in 500 mg l^{-1} IBA, plug trays with Vegro Klay Mix A	100% rooting
Campos & Pais, 1996	*P. indica*	propagation, shoot proliferation	nodal stem from *in vitro* seedling	22 ± 2° C 11.5 W m^{-2} 16 h	BM: MS, 30 g/l sucrose, 8 g/l agar, pH = 7.0 PGR: 1 mg l^{-1} BA	shoot elongation from buds
		propagation, rooting	terminal shoot cutting 2 – 8 cm from shoot cultures		induction: 1–2 s dip in 1–5 mg/ml IBA development: BM with ½ MS medium, no PGR	100 % rooting
Wang & Hu, 1984	*Sassafras randaiense*	Propagation: shoot multiplication	1 cm shoot tips from 5 yr-old grafted trees	18 – 20°C, 2 klx cool white fluorescent light, 12 h	BM (MS salts with 2x FeEDTA and LS organic, 5% sucrose), 30% LCE, 100 mg l^{-1} malt extract, 50 mg l^{-1} glutamine, 50 mg l^{-1} arginine PGR: 5 mg l^{-1} Kin, mg l^{-1}, 0.05 NAA (60 mg l^{-1} Kin required for stage II)	shoot multiplication
		propagation: rooting	*in vitro* shoot ≥ 1 cm		BM PGR: 5 – 10 IBA liquid medium with filter paper support	rooting of ≤ 20%

Table 2-6-continued

Reference	Species, cultivars	Purpose of studies	Explant	Physical environment	Medium[z]	Responses
Protoplast Isolation						
Blickle et al., 1988	*P. americana* cv. was not reported	protoplast isolation	stem-derived callus: 500 mg		Preplasmolyzation for 45 min in solution: 10 ml of 0.7 M mannitol, 0.01 M MES, 0.4% PEG 6000, MS salts, pH 5.3 Digestion in preplasmolyzation solution and 1% Cellulase Onozuka RS and 0.1% Pectolyase Y23 for 3 h at 50 rpm at 29°C	$2 - 3x\ 10^6$ protoplast g^{-1} callus
Percival et al., 1991	*P. americana* 'Hass"	ripening studies	mesocarp from mature fruits	not applicable	Predigestion solution (45 min): 0.6 M mannitol, 0.5% BSA0.1 mM dithiothreitol and mineral salts (Patnaik et al., 1981). Digestion solution (overnight, 18 h): predigestion solution, 1% Rhozyme HP-150, 1% Cellulysin, 0.5% Macerase	protoplast yield of $2 - 6x10^6$ per gram tissue

[z] BM = basal medium, MS = Murashige & Skoog (1962), WPM = Woody Plant Medium (Lloyd & McCown, 1980), LCE = liquid coconut endosperm, MES = morpholino ethane sulfoxide, PEG = polyethylene glycol

PGR = plant growth regulator in µM except stated otherwise, IAA = indoleacetic acid, NAA = naphthaleneacetic acid, IBA = indolebutyric acid, 2,4-D = 2, 4-dichlorophenoxyacetic acid, BA = benzyladenine, 2iP = 2-isopentenyladenosine , Kin = kinetin, GA_3 = Gibberellic acid

and *P. indica* (Aaouine, 1986) and cotyledons of *P. palustris* (Raf.) Sarg. (Kane et al., 1989). Regardless of the objectives of these studies, shoot organogenesis was never reported.

Schroeder (1980) attempted to initiate shoot growth and proliferation of shoot cultures of avocado; however, shoot growth was poor on MS medium. Suboptimal growth responses, i.e., die back and leaf abscission (Pliego-Alfaro, 1981; Pliego-Alfaro et al., 1987), scaly leaf formation (Kane et al., 1989: Gonzales-Rosa et al., 1985), extensive callus formation at the base of explants (Schroeder, 1980; Gonzales-Rosas et al., 1985) and survival rate <100% (Gonzales-Rosas & Salazar-Garcia, 1984; Schall, 1987) were reported. Better proliferation has been achieved mostly by reducing the major salts content in MS medium (Schall, 1987; Nel et al., 1982; Campos & Pais, 1996). Variable rooting frequencies have been reported (Table 2-6) from 100% (Pliego-Alfaro, 1988; Kane et al., 1986; Campos & Pais, 1996) to 30% (Skene & Barlass, 1983; Berringer et al., 1996). The difference in rooting frequency may be genotype-dependent or related to degree of juvenility and rooting method. Nevertheless, tissue culture propagation and plant establishment in soil have been reported for commercial avocado and several species belonging to *Lauraceae* (Table 2-6), although the reported procedures have involved juvenile phase explants. Therefore, the practical application of this technique is limited to propagation of germplasm for breeding programs or propagation of endangered species (Campos & Pais, 1996). Attempts to culture adult materials have been unsuccessful (Pliego-Alfaro, 1981), although limited shoot proliferation followed by low rooting frequencies can be obtained from adult phase shoot cultures that have been partially rejuvenated (Pliego-Alfaro & Murashige, 1987; Schall, 1987).

Somatic embryogenesis derived from zygotic embryos has been reported, although plant recovery has been only 2.5-5% (Mooney & Van Staden, 1987; Pliego-Alfaro & Murashige, 1988). Only three genotypes have been regenerated, and suspension cultures were not obtained. These reports are therefore largely preliminary.

Avocado protoplasts have been isolated from callus (Blickle et al., 1988), in order to study sunblotch viroid replication, and from fruit mesocarp (Percival et al., 1991), for studying fruit ripening, Consequently, morphogenic responses of protoplasts have not been reported.

Somatic Embryogenesis

Since the first description of somatic embryogenesis by Reinert (1958) and Steward (1958), this regeneration pathway has been widely used as a tool to study plant development (Zimmerman, 1993), for clonal plant propagation and for development of technology for plant improvement through somatic cell genetics and genetic transformation.

Somatic embryogenesis refers to the developmental pathway from somatic cells/tissues that mimics zygotic embryogenesis (Zimmerman, 1993). From a practical standpoint, somatic embryogenesis is a process whereby embryos develop from somatic cells/tissues in tissue culture and can develop to maturity and subsequently germinate, forming normal plants (Wann, 1988). Low frequency of root emergence and even lower shoot development from somatic embryos is common in many species. In the view of many, the concept of plant conversion is therefore more operational than germination for *in vitro* somatic embryogenesis (Redenbaugh, 1993). Germination in this dissertation is defined as the development of roots from somatic embryos, while plant conversion is defined as the development of both shoots and roots from the same somatic embryo regardless of the plantlets survival during acclimatization and production two new leaves as required under Redenbaugh's criteria (1993).

Somatic embryogenesis can be divided into several stages:

1. initiation of embryogenic culture,
2. maintenance of embryogenic culture,

3. somatic embryo development or maturation, and
4. somatic embryo germination or conversion.

Initiation of Embryogenic Cultures

Somatic embryos can develop from single cells within an explant (direct somatic embryogenesis), or differentiation can be preceded by proliferation of callus cells (indirect somatic embryogenesis). In the indirect pathway, cells undergo a directive induction event that involves a change of competence. Competent cells undergo a permissive induction that results in the embryogenic pathway. In directive induction, embryogenically predetermined cells simply require permissive induction to express the embryogenic pathway, e.g., cells in explanted nucellar cultures of polyembryonic plants (Ammirato, 1987).

Indirect Pathway

In the indirect pathway, differentiated explants, i.e., leaves, flower buds, hypocotyls, etc., are explanted onto inductive plant growth medium containing auxin (usually 2,4-D) in order to obtain an embryogenic culture. Embryogenic cultures consist of different cell types: parenchymatous, elongated and vacuolated cells, highly cytoplasmic isodiametric cells and cell clusters that resemble proembryos (Halperin, 1966). In the presence of auxin, cells within the rapidly dividing cell population are stimulated to undergo unequal divisions. This results in the formation of two sister cells: a large, highly vacuolated cell and a small, cytoplasmically rich cell that is competent for embryogenesis (Litz & Gray, 1995). Unequal cell division or segmentation of elongated cells has been documented in carrot (Backs-Hüseman & Reinert, 1970; Street & Withers, 1974), *Salix* (Grönroos, 1995) and *Coffea canephora* (P. ex Fr.) (Berthouly & Michaux-Ferriere, 1996). The competent cells form proembryonic cell clusters. Subculture of embryogenic cultures on or in induction medium results in the loss of integrative organization of the

proembryos. The embryogenic masses that develop from proembryos in the presence of 2,4-D are proembryonic or proembryogenic. More than one somatic embryo can develop from each proembryonic mass when it is transferred to 2,4-D-free medium (Halperin, 1966). In tropical woody species, the indirect pathway of somatic embryogenesis has been reported to occur from nucellar explants of monoembryonic mango species (Litz et al., 1982), and from leaf explants of coffee (Sondahl & Sharp, 1977) and longan (Litz, 1988).

Direct Pathway

In the direct pathway, somatic embryos or proembryonic masses can develop from the explant (immature zygotic embryo, nucellus, hypocotyl, etc.) without a callus phase. Somatic embryos or proembryonic masses can be initiated from embryogenic cells that are present in the explant. In pearl millet, Vasil and Vasil (1980) demonstrated that somatic embryos can originate from subepidermal cells of the scutellum of immature embryos. In the presence of 2,4-D, those scutellar cells enlarge and undergo internal segmentation resulting in the formation of proembryos. In polyembryonic mango, the embryogenic cells that are already present in nucellar tissue are similar morphologically to proembryos (DeWald, 1987). Auxin in the medium can stimulate the cloning of such cells (Carman, 1990; Wann, 1990; Ammirato, 1985). Direct development of disorganized proembryonic masses or somatic embryos from carrot explants can be controlled by manipulating medium pH (Smith & Krikorian, 1990).

Induction of the direct embryogenic pathway in woody perennials has been reported from the nucellus of polyembryonic mango (Litz, 1984; DeWald, 1987; Litz et al., 1995) and monoembryonic mango (Jana et al., 1994); cotyledons of wild pear (*Prunus avium*) (De March et al.,1993); and nucellus of monoembryonic citrus such as *Citrus grandis* Osbeck, *C. limon* Burmf. and *C. reticulata* Blanco x *C. sinensis* Osbeck (Rangan et al.,1969).

Maintenance of Embryogenic Cultures and Morphological Variability of Proembryonic Masses

Embryogenic cultures are subcultured as proembryonic masses and not callus (Litz & Gray, 1992). Proliferation of proembryonic masses (and somatic embryos) has been referred to as repetitive embryogenesis (Ammirato, 1987; Litz & Gray, 1992). Repetitive embryogenesis involves continuous cycles of secondary somatic embryogenesis from proembryonic masses that have lost their integrative ability to form single somatic embryos (Williams & Maheswaran, 1986). This has been described with walnut (Preece, 1995), big leaf magnolia (Merkle & Watson-Pauly, 1993), mango (Litz et al., 1995) and citrus (Button et al., 1974; Cabasson et al., 1995; Gavish et al., 1991)

The loss of integrative development at the proembryo stage may result in the continuous proliferation of proembryos to form disorganized proembryonic masses, i.e., citrus (Cabasson et al., 1995). The loss of integrative development at the globular to heart stage may result in the proliferation of organized proembryonic masses, i.e., mango. The cells of the protoderm and subepidermal layer become embryogenic and may slough off from the primary proembryos (Button et al., 1974).

Repetitive somatic embryogenesis by production of singulated maturing somatic embryos usually is slow (Gray, 1995; Merkle, 1995) and not as amenable to genetic manipulation through genetic transformation and to mass propagation as proliferation of embryogenic cultures through proembryonic masses (Merkle, 1995). Therefore repetitive embryogenesis through proembryonic masses proliferation is desirable, not only for maintenance of cultures but for somatic cell genetics.

Auxin is one of the most significant inductive factors for embryogenic cultures, and because of its ability to inhibit embryo development, it is often critical for maintenance of embryogenic cultures (Litz & Gray, 1992). Auxin can disrupt the polarized cell division that is required for integrated development of proembryos to somatic embryos (Kawahara

& Komamine, 1995). Habituation may also occur, e. g., in citrus proembryonic masses (Button & Kochba, 1974; Grosser & Gmitter, 1990).

Proliferation of proembryonic masses has been reported to be affected by genotype in mango (Litz et al., 1995) and sweet gum (Merkle, 1995a). Very often, a whole range of different sizes and morphologies of proembryonic masses and somatic embryos is present in cultures of different species of sweet gum (*Liquidambar styracifolia*) and proembryonic masses can be preferentially selected for further subculture (Merkle, 1995a). Medium pH can affect the proliferation of wild carrot cultures, so that low pH favors the proliferation of proembryonic masses while high pH favors the proliferation and development of somatic embryos (Smith & Krikorian, 1990).

The total nitrogen concentration and the ratio of NH_4^+ : NO_3^- have been reported to affect proembryonic mass proliferation. In citrus, total nitrogen concentration of 60 mM nitrogen resulted in significantly higher proembryonic mass fresh weight compared to 30 mM, while high fresh weight was maintained with a NH_4^+ : NO_3^- ratio of 1:1 to 1:9 (Niedz, 1993).

Somatic Embryo Development

Somatic embryo development is comparable to that of zygotic embryos, and includes distinct morphological stages, e.g., globular, heart, torpedo and cotyledonary. In addition, formation of morphologically abnormal somatic embryos has been reported. Morphological anomalies include monocotyly, polycotyly, fused cotyledons, fasciation, and multiple somatic embryos (Ammirato, 1987; Litz et al., 1995; Gray, 1995). Sieving and fractionation followed by culturing small polyembryonic masses on somatic embryo development medium has been used to reduce the frequency of multiple somatic embryos and to increase the frequency of singulated somatic embryo (Ammirato, 1987; Litz et al., 1995; Merkle, 1995b).

Somatic embryo development is initiated after transfer of embryogenic cultures to medium without or with low auxin content. Fujiwara & Komamine (1975) observed that cytokinins do not affect development of somatic embryos, although development of shoot meristems is enhanced. Abscisic acid has been demonstrated to affect the differentiation of apical meristems and subsequent plant conversion (Nickel & Yeung, 1993).

Sucrose at concentrations of 1–6% is generally used for somatic embryo development. Replacing sucrose with another carbon source, e.g., glycerol (Ben-Hayyim & Neuman, 1983; Gavish et al., 1991; Vu et al., 1993), galactose (Cabasson et al., 1995), lactose and raffinose has been reported (Kochba et al., 1978; 1982).

The effect of the mineral composition of the maturation medium has been documented. DeWald et al. (1989a) demonstrated that medium based upon a modification of B5 formulation resulted in better mango somatic embryo development than MS. Similar results were reported by Muralidharan & Mascarenhas (1995) with *Eucalyptus citriodora*.

Incomplete maturation of somatic embryos is one of the most significant factors that account for low rates of plant conversion (Bornman, 1992). Precocious germination of immature *Hevea brasiliensis* Müll. Arg. somatic embryos has resulted in poor plant regeneration (Michaux-Ferrière et al., 1991). Somatic embryo maturation can be controlled by treatments with ABA, sucrose and desiccation. Abscisic acid has been used to prevent precocious germination (Ammirato, 1985), to inhibit secondary somatic embryogenesis (Monsalud et al., 1995), and to confer desiccation tolerance in orthodox type somatic embryos (Kim & Janick, 1989), thereby promoting accumulation of storage lipids, starch and proteins, synchronous maturation and a high frequency of conversion (Bornman, 1992). Nickel & Yeung (1993) suggested that ABA may induce shoot meristem differentiation or prevent precocious germination that includes precocious vacuolation of the cells in the apical notch that prevents meristem differentiation. Although application of ABA together with an osmoticum can increase plant conversion

of conifers (Attree & Fowke, 1993), this approach has been unsuccessful for angiosperm tree species (Merkle, 1995b; Vieitez, 1995; Pliego-Alfaro & Murashige, 1988).

Pence (1991) demonstrated that during maturation of recalcitrant zygotic embryos of *Theobroma cacao*, ABA levels increased and peaked at the beginning of maturation and then dropped subsequently. Water content of the embryo also decreased ca. 40–70% and synthesis of anthocyanins and lipids increased up to the end of maturation period. Similar trends were observed by Etienne et al. (1993) with recalcitrant *H. brasiliensis* zygotic embryos, while somatic embryos had low ABA content, which varied little during maturation. Inclusion of ABA in maturation medium, however, did not significantly affect desiccation tolerance of either cacao zygotic embryos (Pence, 1992) or mango somatic and nucellar embryos (Pliego-Alfaro et al., 1995a; b), although Etienne et al. (1993) claimed that high osmolarity (100 g/l sucrose) together with high levels of ABA (1 mM) dramatically improved *H. basiliensis* somatic embryo desiccation tolerance and resulted in 77% germination and 34% conversion.

Slow desiccation has been demonstrated to improve maturation of *H. brasiliensis* somatic embryos (Etienne et al., 1993). In walnut, desiccated orthodox somatic embryos convert at a high rate (45%) (Deng & Cornu, 1992).

Merkle (1995a; b) was able to prevent precocious germination of yellow poplar somatic embryos by allowing their development on filter paper overlain on semisolid medium. These somatic embryos matured and converted to plantlets at frequency up to 100% upon transfer to germination medium. Filter paper may impose a matrix stress on somatic embryos that encourages maturation (Merkle, 1995a, 1995b).

Somatic Embryo Germination and Plant Conversion

Various treatments have been demonstrated to increase shoot development/plant conversion from somatic embryos of woody plants, including localized application of

cytokinin (Mathews & Wetztein, 1993), cold treatment (Arilaga et al., 1994), gibberellic acid (Deng & Cornu, 1992) and high CO_2 together with high irradiance (Figueira & Janick, 1993).

Cold treatment increased plant conversion of (orthodox) black locust somatic embryos (Arrilaga et al., 1994), walnut (Deng & Cornu, 1992), chesnut (Vieitez F, 1995), *Camelia japonica* and *C. reticulata* (Vieitez A, 1995), although recalcitrant avocado somatic embryos did not respond (Pliego-Alfaro & Murashige, 1988). Cold treatment may be essential for species that require cold stratification for optimum germination, i.e., temperate hardwood species with orthodox embryos (Merkle, 1995b).

Mathews and Wetzstein (1993) applied 100 μM BA and 3 mg l-1 anti ethylene silver nitrate topically to orthodox pecan somatic embryos (*Carya illinoensis*) and increased germination from 3 to 47% and plant conversion from 2 to 13%. Gibberellic acid as a component in germination medium has increased the conversion rate of orthodox walnut somatic embryos with or without a cold treatment; however, the conversion rate of desiccated walnut somatic embryos was reduced (Deng & Cornu, 1992). Gibberellic acid in combination with IAA increased the conversion rate of orthodox *Camellia japonica* and *C. reticulata* in comparison with gibberellic acid alone (Vieitez A, 1995). The regulation of orthodox seed germination by gibberellic acid has been widely used in cereals (Berrie, 1984), but its effects are not understood (Deng & Cornu, 1992).

Maintaining recalcitrant somatic embryos of cacao under high CO_2 (20,000 ppm) and high irradiance (159–299 μmol m^{-2} s^{-2}) (Figuera & Janick, 1993) has been demonstrated to increase the conversion and vigor of regenerated plantlets. The importance of high CO_2 and high irradiance has been to stimulate photoautotrophy through the promotion of the growth of chlorophyllous explants/plantlets (Kozai, 1991).

In conclusion, somatic embryogenesis has been widely reported and studied for different plant species, and has great potential for clonal propagation and crop improvement. It is necessary to solve problems related to developmental anomalies that

are associated with this developmental pathway, such as poor embryo organization, embryo maturation and somatic embryo germination or plant conversion. Some of the problems have been solved by providing conditions that mimic the *in vivo* condition of the zygotic embryo. For recalcitrant species, e. g., mango, cocoa and avocado, however, their seed physiology is not widely understood. Consequently, attempts to resolve problems associated with embryo development and maturation unavoidably are based on orthodox seeds/somatic embryos and therefore are often ineffective.

Protoplast Isolation, Culture and Regeneration

Protoplast technology has been important in somatic cell genetics for genetic transformation and for somatic hybridization. The application of somatic cell genetics to woody fruit species is especially important for supplementing conventional plant breeding. Discrete genetic variability could be directly selected without prolonged crossing and backcrossing because woody fruit species are propagated vegetatively (Janick, 1992). The production of somatic hybrids involving citrus and its relatives by protoplast fusion can involve sexually incompatible species belonging to different genera or tribes (Grosser et al., 1992).

The potential of somatic hybridization requires the availability of an efficient plant regeneration system from protoplasts. Among woody plants, an efficient protoplast-to-plant regeneration system was first reported for citrus (Vardi et al., 1972). Plants have also been regenerated from protoplasts of sandalwood (*Santalum album* L.) (Rao & Ozias-Akins, 1985), from leaf protoplasts of species in the family Rosaceae, including *Malus*, *Prunus* and *Pyrus* (Ochatt, 1990;1993b, c; Ochatt et al., 1992; Patat-Ochatt, 1993), and of species such as grapevine (Reustle et al., 1995), *Actinidia chinensis* (Tsai et al., 1993) and *Passiflora* spp. (Dornelas et al., 1993; De'Utra Vaz et al., 1993).

Protoplast Isolation

The discussion that follows is intended as a review of those factors that affect isolation and culture of protoplasts together with plant regeneration, particularly with respect to woody perennials and fruit trees.

Protoplasts can be isolated either mechanically or enzymatically. For *in vitro* studies, the latter method has been preferred, because a large number of protoplasts can be released from various tissues (Cocking, 1972). The use of enzymes for protoplast isolation was pioneered by Cocking in 1960 who isolated protoplasts from tomato roots following digestion with cellulase purified from a fungus, *Myrothecium verrucaria* (Cocking, 1983).

Enzymes

The enzyme mixture that is utilized for protoplast isolation generally contains pectinase for loosening the tissue and cellulase for degrading the cell wall. Various enzymes are available commercially with different levels of purity (Table 2-7). Impure enzyme preparations contain nucleases (especially ribonucleases), peroxidases, proteolytic enzymes and phenolic compounds (Vasil & Vasil, 1980). Partial purification of commercially available enzyme precipitation with ammonium sulfate and elution through Sephadex G-25 or Biogel is sometimes useful (Vasil & Vasil, 1980). Very highly purified and crystalline enzymes are not useful since they are unable to digest complex plant cell walls; therefore complex enzyme mixtures, cleansed of toxic substance and impurities are recommended (Vasil & Vasil, 1980). Purified enzymes such as Cellulase Onozuka RS, Cellulase Onozuka R10, macerozyme R10 and Pectolyase Y23 have been recommended for protoplast isolation from tissues of woody plants (Grosser & Gmitter, 1990; Ochatt et al., 1992b). For the most part, enzymes for woody plant protoplast isolation have been used at concentrations of 0.3–2%, except for Pectolyase Y23 which is generally used at

Table 2-7. Enzymes used for protoplast isolation.

Enzyme	Concentration in culture	Purity	Organismal source	Commercial source
Cellulase				
Cellulase Onozuka R-10	1.0–3.0		*Trichoderma viride*	Yakult Honsha Co, Tokyo Karlan Chemical, Santa Rosa, CA
Cellulase Onozuka RS	1.0	high	*T. viride*	Yakult Honsha Co Tokyo, Japan Karlan Chemical, Santa Rosa, CA
Meicelase P-1				Meiji Seika Kaisha, Tokyo, Japan
Meicelase	1.0–4.0			
Driselase		crude	*Basidiomycetes*	Sigma
Driselase	1.0–2.5		*Irpex lacteus*	Kyowa Hakko Kogyo, Tokyo,
Cellulase		crude	*T. viride*	Sigma Chemical Co. St. Louis, MO, USA
Cellulase YC			*T. viride*	Seishin Pharmaceutical Co., Tokyo
Cellulysin	1.0–3.0		*T. viride*	Calbiochem, San Diego, CA
Cellulase CEL		high	*T. viride*	Cooper Biomedical Inc., Malvern, PA
Hemicellulase				
Helicase			*Helix pomatia*	Industrie Biologique Française, Genevilliers, France
Hemicellulase	0.2–0.5	crude	*Aspergilus niger*	Sigma Chemical Co. St. Louis, MO
Rhozyme HP150	1.0–2.0		*Aspergilus niger*	Genencor Inc., South San Franscisco, CA. Corning Glass, Corning, NY

Table 2-7—continued

Enzyme	Concentration in culture	Purity	Organismal source	Commercial source
Pectinases				
Macerozyme R-10	0.5–1.0	high	*Rhizopus arrhizus*	Yakult Honsha Co, Tokyo Karlan Chemical, Santa Rosa, CA
Pectolyase Y-23	0.1	high	*Aspergillus japonicus*	Seishin Pharmaceutical Co., Tokyo Karlan Chemical, Santa Rosa, CA
Macerase (macerozyme)	0.5–1.0		*Rhizopus arrhizus*	Calbiochem, San Diego, CA, USA
Pectinase	0.5–1.0		*Aspergilus niger*	Sigma Chemical Co. St. Louis, MO, USA
PATE (Pectic - acid- acetyl-transferase)	0.1		*Bacillus polymixa*	Farbwerke-Hoechst AG, Frankfurt, FRG
Pectolyase		crude	*A. japonicum*	Sigma Chemical Co. St. Louis, MO, USA

Sources: Ishii (1989); Evans and Bravo (1983)

10% of the concentration of cellulase. A mixture of enzymes is normally used. Since cell wall composition of different tissues of different species is variable (Ishii, 1989), the enzyme composition of the digestion solution may vary and must be determined empirically for different species (Cocking, 1972; Vasil & Vasil, 1980; Evans & Bravo, 1983; Davey & Kumar, 1983). Ishii (1989) demonstrated that the cell wall composition of monocotyledonous and dicotyledonous species was different, and demonstrated that xylanase is required for optimum protoplast release from oat leaves while pectin lyase was required for optimum protoplast release from petunia leaves. With woody species, a single enzyme mixture may be effective for several species, although slight variations may be required, depending on the tissue (Ochatt, 1990). A mixture of 1% Cellulase Onozuka R10, 1% Hemicellulase and 0.1% Pectolyase Y23 has been used for protoplast isolation from numerous fruit and nut genotypes (Revilla et al., 1987; Ochatt, 1990; 1993b), while 2% Meicelase, 2% Rhozyme HP1500 and 0.03% Macerozyme R10 has been used to isolate protoplasts from suspension cultures and callus tissues of cherry, pear and apple (Ochatt, 1990; 1993a, b). An enzyme mixture that was developed initially for *Trifolium rubens* (Grosser & Collins, 1984) and improved for citrus contains 1% Cellulase Onozuka RS, 1% Macerase or macerozyme R10 and 0.2% Pectolyase Y23 (Grosser & Gmitter, 1990) and is effective for various tissues and species, including embryogenic cultures and suspension cultures of citrus, non-embryogenic callus and suspension culture of citrus and citrus relatives (Muorao-Filho & Grosser, 1992; Grosser & Gmitter, 1992), leaves from *in vitro* and greenhouse grown citrus and citrus relatives (Grosser & Chandler, 1987), flower bud tetrads, and for other species, including embryogenic suspension cultures of mango and grape (Grosser, 1993, personal communication).

Other components of enzyme mixtures

To prevent protoplasts from bursting after release, increased osmolarity of the medium is essential. Mannitol (0.7 M) has generally been used satisfactorily for citrus

(Grosser & Chandler, 1987; Grosser & Gmitter, 1990; Kobayashi et al., 1985; Sim et al., 1988) and *Actinidia chinensis* (Tsai, 1993) even though lower concentrations have been used for protoplast isolation from grape embryogenic cultures (Reustle et al., 1995).

A minimal salt mixture has been included in the enzyme mixture to reduce shock following transfer of protoplasts to culture medium. These media include CPW salts (Frearson et al., 1973) and 0.5 x MT (Murashige & Tucker, 1965) (Kobayashi et al., 1985; Sim et al., 1988).

Other addenda such as morpholinoethanesulfoxide (MES) at concentrations of 3–7 mM (Grosser & Gmitter, 1990; Ochatt, 1990; 1993a, b) has been used to buffer the acidification that often occurs during digestion. Polyvinylpyrollidone (PVP) has been used to prevent phenolic oxidation, especially when protoplast isolation involves leaf or suspension cultures of woody perennials (Ochatt, 1993a, b; Patat-Ochatt, 1993; Lee & Wetzstein, 1994). Other substances, i.e., $CaCl_2$ and NaH_2PO_4, are added as plasma membrane stabilizers of protoplasts (Vasil & Vasil 1984; Grosser & Gmitter, 1990).

Other physical factors influencing protoplast isolation

Enhancement of enzyme penetration into tissue is usually required when clumps of tissues or leaves are used. With herbaceaous plants, this can be achieved by removing the epidermis and chopping the tissues into small pieces (Evans & Bravo, 1983). With woody species, the epidermis is difficult to remove; therefore, leaf tissue can be chopped or feathered (Grosser & Gmitter, 1990). When leaf tissue is derived from greenhouse-grown plants, vacuum infiltration may also facilitate enzyme penetration (Grosser & Gmitter, 1990). Grinding of leaves can provide a larger surface area for enzyme digestion, while entrapment of protoplasts during purification can be prevented by washing the cell debri (Russel & McCown, 1986), agitation of the digestion mixture and running the digested mixture up and down a Pasteur pipet (Mills & Hammerschlag, 1994).

The ratio of tissue:enzyme solution (w/v) can be critical for protoplast isolation from peach leaves where ratios of 10–20 mg ml^{-1} resulted in high protoplast yields while ratios of 50–100 mg ml^{-1} resulted in low protoplast yields (Mills & Hammerschlag, 1994). High tissue:enzyme ratios, however, have been used for other tissues, i.e., 100 mg ml^{-1} for pear leaves (Ochatt & Power, 1988), and 150 mg ml^{-1} for apple leaves (Doughty & Power, 1988). Grosser & Gmitter (1990) used a tissue:enzyme ratio of ca. 250 mg ml^{-1} for embryogenic citrus cultures, and 30–50 mg ml^{-1} for leaves. The negative effect of higher tissue:enzyme ratios may be associated with release of protease from digested tissues that inhibits enzyme activity (Mills & Hammerschlag, 1994) and phenolic compounds. The tissue:enzyme ratio may be genotype and tissue dependent.

Protoplast purification

Protoplasts are generally purified by passing the digestion mixture through a nylon or stainless steel sieve (45–90 μm mesh) or another material, e.g., Miracloth®. This is sometimes followed by gradient centrifugation to remove cell debris, undigested cells and broken protoplasts that can reduce culture pH, protoplast viability and fusion frequency (Grosser, 1994). Grosser & Gmitter (1990) have sieved with a 45 μm stainless steel mesh screen followed by gradient centrifugation at 100 g in 0.7 M (25% w/v) sucrose and 0.7 M (13% w/v) mannitol supplemented with modified CPW salts (Frearson et al., 1973). The purified protoplasts in the interphase can be washed again (Grosser & Gmitter, 1990).

Protoplast Culture

Factors affecting protoplast culture and subsequent growth and development include medium composition, osmoticum and osmolarity, plating density and environmental conditions, i.e., irradiance and temperature (Dons & Colijn-Hooymans, 1989). These factors can be optimized on the basis of plating efficiency, i.e., the proportion of protoplasts that respond to culture conditions by undergoing division after a determined time period.

Medium composition

Protoplast culture media are generally composed of major and minor salts, vitamins and other organic addenda, plant growth substances, a carbon source and an osmoticum. Plant growth media that have been developed for a particular genotype can be used (Davey & Kumar, 1983). This approach seems logical and efficient even though some modification of the medium composition is sometimes needed.

Major and minor salts of MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1965) and KM8 or KM8P (Kao & Michayluk, 1975) media are satisfactory. The success of the KM8P medium may be due to the presence of multivitamins, sugar and sugar alcohol additives that provide essential metabolic intermediates (Grosser, 1994). An efficient protoplast culture medium for species within a genus can be developed by supplementing optimal basal media that have been effective for cell culture of the genus with 8P multivitamins, organic acid and sugar/alcohol additives (Grosser, 1994). This approach has been successful for *Trifolium* (Grosser & Collins, 1984), citrus (Grosser & Gmitter, 1990; Niedz, 1993) and apple (Kouider et al., 1984; Deng et al., 1995; Doughty & Power, 1988; Patat-Ochatt et al.,1993). Ochatt & Power (1988), however, reported that pear (*Pyrus communis* L.) mesophyll protoplasts only regenerate cell walls in K8P or KM8P or MS media, but divide in MS medium without ammonium. The negative effect of K8P medium on protoplast division has also been reported for root callus protoplasts of sour cherry (*Prunus cerasus* L.) (Ochatt, 1990). Ochatt (1993a, b) suggested that scion and rootstock genotypes may require different organic additives, i.e., rootstock genotypes require more organic compounds than scion genotypes in the same genus, i.e., *Pyrus* and *Malus* (Ochatt et al., 1992b).

The ammonium nitrate content of KM8P medium may be deleterious to protoplasts (Grosser, 1994). Elimination of NH_4NO_3 from culture medium has been important for microcallus formation of pear (*Pyrus* sp.) (Ochatt 1990; 1992; 1993; Ochatt & Power, 1988a, b; Ochatt & Caso, 1986), for plant regeneration from protoplasts of

Populus spp. (Russel & McCown, 1986; 1988) and for somatic embryogenesis and subsequent plant regeneration from protoplasts of citrus (Grosser & Gmitter, 1990; Niedtz, 1993).

Plant growth substances, i.e., cytokinin (BA, zeatin) and auxin (2,4 D, NAA) are required for microcallus development when protoplasts are isolated from leaves, callus or suspension culture of pear (Ochatt, 1990; 1993b), *Prunus* spp.(1990; 1993c), apple (Patat-Ochatt et al., 1993), cell suspensions of *Vitis labruscana* and *V. thunbergii* (Mii et al., 1991) and *in vitro* leaves of *Vitis* spp. (Lee & Wetztein, 1988), callus of kiwifruit (Oliveira & Pais, 1991), leaves from greenhouse-grown passionfruit (d'Utra Vaz et al., 1993) and peach suspension cultures (Matsuta et al., 1986). Although sometimes also required for microcallus development from protoplasts isolated from embryogenic cultures of *Vitis* sp. (Reustle et al., 1995), plant growth substances are not generally required for somatic embryogenesis of protoplasts isolated from embryogenic cultures, e.g., citrus (Grosser & Gmitter, 1990; Niedz, 1993; Kunitake et al., 1991; Kobayashi et al., 1985; Hidaka & Kajiura, 1988; Sim et al., 1988; Vardi & Galun, 1988; 1989). Protoplasts isolated from embryogenic cultures derived from seedling parts of citrus relatives such as *Citropsis schweinfurthii*, *Atalantia biloculoaris*, etc. reportedly required plant growth substances such as BA or GA_{4+7} for microcallus formation (Jumin & Nito, 1995); however, protoplasts from similar tissues of *Microcitrus* did not require plant growth substances (Vardi et al., 1986). The plant growth regulator requirement for protoplast growth and development is probably determined by whether regeneration from the source is by organogenesis or somatic embryogenesis.

Plating Density

Protoplasts of woody species are generally cultured at plating densities of 0.5–1 x 10^5 protoplasts ml^{-1}, which is considered optimum for citrus (Vardi & Galun, 1988; 1989;

Grosser & Gmitter, 1990), *Prunus* spp. (Ochatt, 1993c), *Pyrus* spp. (Ochatt, 1993b), *Malus* sp. (Patat-Ochatt, 1993), kiwifruit (Oliveira & Pais, 1991), *Passiflora* spp. (Dornelas & Vieira, 1993; d'Utra Vaz, 1993) and *Vitis* spp. (Mii et al., 1991).

Protoplast plating density affects plating efficiency in apple (Kouider et al., 1984) and root callus protoplasts of sour cherry (*Prunus cerasus* L.) (Ochatt, 1990). The highest plating efficiency was used to determine the optimum plating density. Determining plating density on the basis of the highest plating efficiency may not be appropriate when regeneration is via somatic embryogenesis, e.g., citrus (Kobayashi et al., 1985). Citrus protoplasts derived from embryogenic cultures and plated at a high plating density (ca. 2 x 10^5) resulted in a high plating efficiency (40%); however, only microcallus formed (Kobayashi et al., 1985). At a low plating density (ca. 2 x 10^4), low plating efficiency resulted (5%); however, somatic embryogenesis occurred. Somatic embryogenesis from citrus protoplasts isolated from embryogenic cultures cultured at low plating density was also reported by Hidaka and Kajiura (1988). Inhibition of somatic embryogenesis when fused citrus protoplasts were grown at a high plating efficiency was also reported (Grosser & Gmitter, 1990; Grosser, 1994).

Medium osmolarity

Medium osmolarity generally is 0.4 to 0.7M and supplied with a metabolically active sugar (sucrose, glucose, or sorbitol) or a metabolically inert sugar such as mannitol. Combining metabolically active and inert sugars has been recommended since the former is metabolized by the growing protoplasts and therefore the medium osmolarity is gradually reduced. This would subsequently reduce shock when the protoplast-derived cell colonies are transferred to regeneration medium (Vasil & Vasil, 1983).

Systematic studies have evaluated the effect of medium osmolarity in factorial combinations with plating density of citrus (*Citrus sinensis*) protoplasts isolated from embryogenic cultures (Kobayashi et al., 1985). The optimum medium osmolarity is

dependent upon protoplast density, although high medium osmolarity (≥ 0.35 M mannitol + 0.15 M sucrose) results in a slightly higher plating efficiency than lower medium osmolarity (< 0.35 M mannitol + 0.15 M sucrose). Low medium osmolarity combined with low plating density resulted in the direct development of somatic embryo from protoplasts, while with high medium osmolarity protoplasts give rise to microcallus. Development of somatic embryos directly from protoplasts cultured at low medium osmolarity (0.04 M sucrose, 0.08 M glucose and 0.23 M mannitol) and low plating efficiency was also reported for *Citrus yuko* (Hidaka & Kajiura, 1988). The development of somatic embryos at low medium osmolarity, however, was inhibited by high plating efficiency. The inhibitory effect of high plating density on somatic embryo development could be overcome by lowering the density (Kobayashi et al., 1985; Grosser & Gmitter, 1990).

Regardless of the positive effect of low medium osmolarity for somatic embryo development, protoplast culture medium has generally been supplemented with high medium osmolarity of ca. 0.6–0.7 M of several different sugars. Grosser & Gmitter (1990) employed 0.6–0.7 M osmoticum that consisted of 0.15–0.25 M sucrose and 0.45 M mannitol, while Vardi and Galun (1989) used 0.3 M sucrose and 0.3 M mannitol. A combination of 0.3 M sucrose and 0.3 M sorbitol was used for culture of protoplasts of *C. madurensis* (Ling et al., 1989) and *C. unshiu* (Ling et al., 1990). Other reports include the use of a only of 0.6 M sorbitol for culture of protoplasts isolated from callus initiated from seedling stems (Jumin & Nito, 1996a; b), 0.15 M sucrose and 0.45 M glucose for embryogenic culture-derived protoplasts of *C. mitis* (Sim et al., 1988) and 0.4 M mannitol, 0.2 M glucose and 0.05 M sucrose for kiwifruit protoplasts (Tsai et al., 1993).

Culture of protoplasts at low medium osmolarity is simpler than culture at high medium osmolarity since the latter requires reduction of medium osmolarity during the culture period (Grosser & Gmitter, 1990; Ochatt, 1993a). The tolerance of protoplasts to low medium osmolarity must be determined.

Method of culture

Protoplasts of woody species have been cultured in: liquid culture as a thin layer, i.e., citrus (Grosser & Gmitter, 1990; Hidaka & Kajiura, 1988; Kobayashi et al., 1985) and pear (Ochatt & Power, 1988a); in liquid culture as a shallow pool, i.e., fused citrus protoplasts (Grosser & Gmitter, 1990) and apple (Ding et al., 1995); immobilized with low melting agarose in disc culture, i.e., sour cherry (Ochatt, 1993b) and semisolid culture for *Passiflora* spp. (Dornelas & Vieira, 1993; D'Utra Vaz et al., 1993), grape (Lee & Wetzstein, 1988) and sour cherry (Ochatt, 1993b); immobilized with agar or gellan gum, i.e., citrus relatives (Jumin & Nito, 1990) and *C. unshiu* (Ling et al., 1991; Kunitake at al., 1991); immobilized in Ca-alginate, e. g., grape (Reustle et al., 1995), apple (Huancaruna-Perales & Schieder, 1993) and citrus (Niedz, 1993); in double phase liquid over semisolid agar/gellan gum, i.e., *C. mitis* (Sim et al., 1988) and *Vitis labruscana* and *V. thunbergii* (Mii et al., 1991).

Liquid culture is easy to handle and facilitates observation with the inverted microscope. When solid culture is required, low melting agarose results in higher plating efficiency (Grosser & Gmitter, 1990), and facilitates protoplasts to grow under low plating density, i.e., 660–1300 protoplasts per ml as demonstrated in *Hyocyamus muticus* and *Nicotiana tabacum* (Shillito et al., 1983). Similarly, agarose has been effective for culturing protoplasts of calamondin (*C. madurensis* Lour.) (Ling et al., 1989). Sour cherry protoplasts, however, had the same plating efficiency regardless of the culture method utilized (Ochatt, 1990), while protoplasts derived from stems and leaves of haploid apple divided in liquid medium, but not in agarose discs or in molten agarose (Patat-Ochatt, 1993). Protoplasts of the shrubby ornamental honeysuckle (*Lonicera nitida* cv. Maigrun) grew better in liquid than in agarose medium (Ochatt, 1991)

Niedz (1993) demonstrated higher plating efficiencies for protoplasts embedded in Ca-alginate beads than in liquid medium when plating densities were high. Oliviera & Pais (1991) observed that callus-derived protoplasts of kiwifruit 'Hayward' did not survive in

liquid medium, but divided and formed microcallus in alginate-solidified medium, although the microcalli eventually died. Ca-alginate has been successfully used to culture protoplasts of embryogenic grape (Reustle et al., 1995).

Culture environment

Protoplast cultures have generally been maintained at 25° C in darkness (Grosser & Gmitter, 1990), since protoplasts can be sensitive to light (Grosser, 1994). Protoplast growth inhibition by light has also been reported for *Lonicera nitida* 'Maigrun' (Ochatt, 1991). Some protoplasts, i.e., sour cherry, can tolerate more light and can grow and differentiate under diffuse light (Ochatt, 1990).

Protoplast culture and regeneration has been reported with several woody species. Regeneration in species other than citrus has mostly been confined to organogenesis since the source of the protoplasts has not been embryogenic tissue. Protoplast culture and regeneration from embryogenic tissues is more efficient and simpler than from nonembryogenic tissues.

Somatic Hybridization

Since the first interspecific somatic hybrids between *Nicotiana glauca* and *N. langdorffii* (Carlson et al., 1972) were reported, somatic hybrids have been produced in various plants, including potato, tomato, legumes, cereals, eggplant, petunia and several trees such as citrus.

Somatic hybridization by protoplast fusion has been utilized to bypass genetic barriers between sexually incompatible wild species and economic species, thereby transferring important traits from wild species into commercial species. Somatic hybrids have been obtained within the genera *Nicotiana*, *Solanum* and *Brassica* (Bajaj, 1994); however, the usefulness of somatic hybrids in breeding has been impeded by sterility and undesirable characters from the wild species. For example, somatic hybrids of eggplant

(*Solanum melongena* L.) with *S. khasianum, S. torvum* and *S. nigrum* were difficult-to-root, grew poorly and were highly sterile, whereas, somatic hybrids between eggplant with more closely related *S. aethiopicum* demonstrated vigorous growth and had high fruit production (Sihachakr et al., 1994). Similar results were reported for somatic hybrids between potato (*Solanum tuberosum*) and related species (Butenko & Kucko, 1994; Jadari et al., 1992). Since the somatic hybrids are tetraploid, their use in breeding of diploid species is dependent on the ability to regenerate diploid plants from microspores and/or intensive backcrossing to the cultivated species (Sihachakr et al., 1994).

Somatic Hybridization and Its Application to Woody Species

The application of somatic hybridization to woody species has been limited to a few genera, mostly *Citrus* spp. and relatives (Grosser & Gmitter, 1990), with which more than 150 somatic hybrids have been produced (Grosser et al., 1996; Grosser, 1997, personal communication). Other somatic hybridizations have involved sexually- and graft-incompatible cherry rootstock 'Colt' (*Prunus avium x Pseudocerasus*) with wild pear (*Pyrus communis* var. *pyraster*) (Ochatt et al., 1989), between sexually compatible *Passiflora edulis* f. *flavicarpa* and *P. incarnata* (Otoni et al., 1995), between *P. edulis* f. *flavicarpa* with *P. alata, P. amethystina, P. cincinata* and *P. giberti* (Dornelas et al., 1995).

Two approaches for somatic hybridization have been used in citrus rootstock improvement. Somatic hybrid rootstocks have been produced from existing rootstocks that have desirable and complementary characteristics. These somatic hybrids would have characteristics of both parents since somatic hybridization is an additive process with no segregation. Therefore, traits that are dominant or codominant should be express in the somatic hybrids. Several somatic hybrids for this purpose have been produced (Gmitter et

al., 1992), although the characterization of the inheritance of the parental characters in the somatic hybrids has not yet been reported.

Another approach involves production of somatic hybrids between existing rootstocks and sexually incompatible, related species for germplasm enhancement. Several somatic hybrids have been produced, including combinations of *C. reticulata* with *Citropsis gilletiana*, and *C. sinensis* 'Hamlin' sweet orange with *Severinia disticha*, *Citropsis gillatiana*, *S. buxifolia*, *Atalantia ceylanica*, *Feronia limonia* and *Clausena lansium*. Somatic hybrids of the last two combinations did not grow vigorously and eventually died. This failure may be due to somatic incompatibility since *C. lansium* and *F. limonia* cannot be grafted easily with citrus (Louzada & Grosser, 1994). Recent attempts involving a vigorous selection of *A. ceylanica* with 'Succari' sweet orange resulted in prolific and more vigorous somatic hybrids (Mourao-Filho, 1995); however, latent somatic incompatibility in these somatic hybrids has not been determined.

Mourao-Filho (1995) reported that somatic hybrid plants between four varieties of sweet orange (*C. sinensis*) + *S. disticha* died following attack by an unidentified fungus, although previously reported somatic hybrids of the same parental combination were reportedly unaffected (Louzada & Grosser, 1994). Analysis of the somatic hybrid plants of 'Cleopatra' mandarin (*C. reticulata*) + *Citropsis gillatiana* showed a high level of susceptibility to an undetermined leaf/stem fungal spotting disease that drastically reduced tree vigor (Mourao-Filho, 1995). It is not clear if this undesirable phenotype was due to somaclonal variation or a negative genomic interaction (Mourao-Filho, 1995).

Grosser et al. (1992) also reported that somatic hybrids between *C. sinensis* 'Hamlin' + *Severenia buxifolia* had chromosome numbers of 27 and it is not clear whether these plants were triploid or aneuploid. Somatic hybridization involving distantly related species can result in asymmetric somatic hybrids due to chromosome elimination (Kao, 1977; Kumar & Cocking, 1987). Somatic hybrids with chromosome number of 24, less

than the allotetraploid (2n = 4x = 32), have been produced from carrot and barley (Kisaka et al., 1997).

Somatic hybridization for citrus scion improvement has been intended for production of seedless triploid scion cultivars either by production of tetraploid somatic hybrids for crosses with diploids or by direct protoplast fusion between diploid and pollen-derived haploid protoplasts (Grosser & Gmitter, 1990).

Somatic hybridization has been reported for producing somatic hybrids between graft- and cross-incompatible cherry rootstock 'Colt' (*Prunus avium x Pseudocerasus*) and wild pear (*Pyrus communis* var. *pyraster*) (Ochatt et al., 1989). Further analysis of the somatic hybrid plants demonstrated that one clone was graft-compatible with both parents (Ochatt & Patat-Ochatt, 1994).

Somatic hybridization has also been used to produce fertile allotetraploid somatic hybrids between *Passiflora edulis* f. *flavicarpa* and *P. incarnata* that have cold tolerance characteristics (Otoni et al., 1995). These somatic hybrid plants are fertile and showed intermediate characteristics of both parents for most characters observed; the natural hybrids of these species are sterile and only their tetraploid (after colchicine treatment) derivatives are fertile. Other somatic hybrids involving *Passiflora* species combinations have been intended to introgress desirable characteristic of wild species into *Passiflora edulis* f. *flavicarpa* (Dornelas et al., 1995).

Somatic hybridization has also been use to produce cytoplasmic hybrids (cybrids). Cybrid production in citrus involves the donor-recipient method (Vardi et al., 1987). Following fusion between protoplasts isolated from embryogenic cultures and from mesophyll protoplasts, diploid plants with morphological characteristics of the leaf parents have been recovered in addition to somatic hybrids (Saito et al., 1993; Tusa et al., 1990; Moriguchi et al., 1996). These plants were cybrids since their mitochondrial DNA was similar to the embryogenic parents and their nuclear DNA was similar to the leaf parent. The acquisition of mitochodrial DNA from the embryogenic parent seems to be a

prerequisite for somatic embryogenesis from non morphogenic leaf mesophyll protoplasts (Grosser et al., 1996). To date there is no application for citrus cybrid production; however, cybridization may increase the availability of citrus germplasm for further somatic fusions (Grosser et al., 1996).

Fusion and Selection Methods and Confirmation of Somatic Hybrids

Protoplast fusion in woody plants is mostly achieved using either the polyethylene glycol (PEG) method or electrofusion. Dextran as a fusion agent was reported for somatic hybridization of *Populus* species (Park & Soon, 1994). PEG has been used to produce more than 150 citrus somatic hybrids (Grosser et al., 1996; Grosser, 1997, personal communication). Electrofusion has been used to produce at least 10 somatic hybrids and cybrids (Saito et al., 1993; Ling & Iwamasa, 1994; Moriguchi et al., 1996; Motomura et al., 1995; Hidaka & Omura, 1992; Yamamoto & Kobayashi, 1995). The widespread use of PEG for somatic hybridization has demonstrated its efficacy and efficiency. Furthermore, it is inexpensive, simple and does not cause protoplast mortality (Grosser & Gmitter, 1990).

Selection of somatic hybrids in citrus involves the use of embryogenic cultures of one parent and non-morphogenic cell suspensions, callus or leaves of the other parent. The embryogenic cells confer regeneration potential to the fused protoplasts while unfused protoplasts of the non-morphogenic parent cannot develop in plant growth regulator-free medium. Habituated nucellar derived embryogenic cultures can be used for one parent in citrus somatic hybridization (Grosser & Gmitter, 1990). Although habituated cultures have lost embryogenic competence, somatic hybrids could be recovered following fusion. It has been possible to regenerate the somatic hybrid alone by using medium with 0.6 M sucrose osmoticum (Ohgawara et al., 1994). This type of selection relies on heterosis- or hybrid vigor-like expression in somatic hybrid cells or embryos, and has also been used to

obtain somatic hybrids between *Prunus* and *Pyrus* (Ochatt et al., 1989) and *Passiflora* (Otoni et al., 1995).

Somatic hybrid identity is generally confirmed by several methods, including morphology characters, chromosome counts and molecular markers, i.e., nuclear ribosomal DNA analysis, mitochondrial DNA analysis (Grosser & Gmitter, 1990) and Polymerase Chain Reaction (PCR)-based Random Amplified Polymorphic DNA (RAPD) analysis. Confirmation of somatic hybridization using RAPD markers in citrus has been shown in some cases to require more than one primer (Mourao-Filho, 1995).

General Conclusion of the Literature Review

Avocado is an important fruit crop; however, breeding new cultivars has been slow and improvement of existing cultivars has not occurred. Biotechnology, i.e., somatic hybridization and genetic transformation, can create genetic variability otherwise unavailable through conventional breeding. Application of these technologies is dependent on the availability of efficient plant regeneration protocols from suspension cultures, protoplasts or other tissues. The most efficient regeneration pathway for woody plants has been somatic embryogenesis. Although preliminary studies regarding somatic embryogenesis of avocado have been reported, the published information has been insufficient to utilize for improving avocado by somatic cell genetics. It is clear that work with other woody species (e.g., citrus, mango, etc.) might be applicable to similar *in vitro* studies with avocado.

CHAPTER 3
INITIATION AND MAINTENANCE OF AVOCADO EMBRYOGENIC CULTURES

Introduction

The avocado (*Persea americana* Mill.) is one of the major fruit crops of the world. The species includes three horticultural races of different climatic adaptation; the tropical *P. americana* var. *americana* (West Indian), the less tropical *P. americana* var. *guatemalensis* (Guatemalan) and the semitropical *P. americana* var. *drymifolia* (Mexican). Despite its great importance, there have been relatively few reports related to cell and tissue culture of this species. The initiation of callus from different plant parts was described by Schroeder (1956; 1961; 1971; 1975; 1978), Blumenfeld & Gazit (1971) and Desjardins (1958); however, these cultures were nonmorphogenic. Embryogenic cultures were initiated from zygotic embryos excised from abscised avocado fruits, but no further somatic embryo development was reported (Barlass & Skene, 1983). Embryogenic cultures were also initiated from early stage 'Hass' (Pliego-Alfaro & Murashige, 1988), 'Fuerte' and 'Duke 7' (Mooney & van Staden, 1987) zygotic embryo explants. Mature somatic embryos were recovered from embryogenic cultures, and low frequency plant recovery was described. The effects of genotype on somatic embryogenesis were not addressed in these reports, and the establishment of embryogenic suspension cultures was not attempted.

Conditions for induction of embryogenic avocado cultures of different genotypes and their growth on semisolid medium and in suspension have been described.

Materials and Methods

Embryogenic Cultures on Semisolid Medium

Induction of embryogenic cultures from zygotic embryos

Avocado fruitlets, 0.3–2.0 cm length without the calyx, representing different cultivars of different races and hybrids involving two or more races were collected from the USDA-ARS Subtropical Horticultural Research Station (Miami, FL) national avocado germplasm repository, the germplasm collection of the University of California, Riverside, CA and the University of Florida, Tropical Research and Education Center (Homestead, FL) (Table 3-1). After removal of sepals and peduncles, the fruitlets were surface-disinfested in a 10–20% solution of commercial bleach containing 10–20 drops of Tween 20® per liter for 10–20 min. Fruitlets were rinsed with two changes of sterile, deionized water. The fruitlets were bisected under axenic conditions. The zygotic embryo was removed from each fruit (Pliego-Alfaro & Murashige, 1988) and transferred onto plant growth medium, i.e., induction medium. A single zygotic embryo was placed into culture in each 60 x 15 mm Petri dish and sealed with Parafilm®. Cultures were maintained in darkness at 25°C. The stage of development of the zygotic embryos and the corresponding fruit size for 'Thomas' are indicated in Table 3-2.

The induction media consisted of 1) MS (Murashige & Skoog, 1962) major salts (MSP) and three modifications of MS, which included 2) omission of NH_4NO_3 (MS$^-$), 3) substitution of $NaNO_3$ (MSNa) for NH_4NO_3 4) KNO_3 (MSK) as the sole nitrogen source (with the concentration of nitrogen being equivalent to that in MS) and 5) B5 major salts (Gamborg et al., 1968). All of the major salts formulations were supplemented with MS minor salts, 0.41 μM (0.1 mg l^{-1}) picloram and (in mg l^{-1}) thiamine HCl (0.4), myo-inositol (100) and sucrose (30,000). The various plant growth media were solidified with 8 g l^{-1} TC agar (Carolina Biological Supply Company).

Table 3-1. Avocado cultivars used for the experiments, their botanical varieties and their sources.

Cultivar	Botanical Variety[z]	Source
Booth 7	G x M	TREC, UF
Booth 8	G x M	TREC,UF
Duke 7	M	UC Riverside
Esther	G x [(G x M) x G]	UC Riverside
Hass	G	UC Riverside
Isham	G	USDA-ARS, Miami
Irwing 56	Complex hybrids	USDA-ARS, Miami
Jose Antonio	W	USDA-ARS, Miami
Lamb	G	UC Riverside
M25864	M	USDA-ARS, Miami
T362	G	UC Riverside
Thomas	M	UC Riverside
Waldin	W	USDA-ARS, Miami
Yon	G x W	USDA-ARS, Miami

[z] From Smith et al. (1992)

Table 3-2. Length of 'Thomas' avocado fruitlet (measured without calyx) in relation to zygotic embryo length, stage of development and morphology.

Fruit Length (cm)	Zygotic Embryo Length (mm)	Developmental Stage	Morphology
<0.5	0.1–0.4	globular	
0.5—0.8	0.5–0.8	early heart stage	
0.9—1.1	1–2	late heart stage	
1.2—1.5	3–7	early torpedo	
>1.5	>7	late torpedo, early cotyledonary stage	

The pH of all media was adjusted to 5.7–5.8 with either KOH or HCl prior to addition of agar and autoclaved at 121°C at 1.1 kg cm^{-2} for 15 min. The plant growth media were dispensed in 10 ml aliquots into sterile disposable Petri dishes (60 x 15 mm). Binomial confidence intervals of 95% were computed for each treatment mean using SAS program (SAS Institute, 1992), and for 0% occurrence, the upper limit of 95% confidence intervals were calculated using "the Rule of Three" (Jovanovic & Levy, 1997)

Induction of embryogenic cultures from nucellar explants

Fruitlets of 0.3–0.5 cm length (without calices) were surface-disinfested as described above. Each fruitlet was bisected longitudinally and the endosperm and the zygotic embryo was removed from each seed. The nucellus together with the integument were removed and plated on the surface of plant growth medium with the nucellus in contact with medium.

This experiment involved 4 genotypes. For 'Thomas', there were two explants from a single ovule in 60 x 15 mm sterile plastic Petri dishes containing induction medium. There were five different induction media whose compositions were described above (see Induction of embryogenic cultures from zygotic embryos); however, for the other genotypes, 'Hass', 'Lamb' and 'T362', six explants from 3 ovules were explanted in 60 x 15 mm sterile plastic Petri dishes containing B5 induction medium. There were total of 36 explants (18 fruitlets) per treatment for 'Thomas', 100 explants (50 fruitlets) for 'Lamb', 412 explant (206 fruitlets) and 36 explant (18 fruitlets) for 'T362'. Percent responses were calculated based on the number of fruitlets, not on the number of explants used for the experiments

Maintenance of embryogenic cultures on semisolid medium

Embryogenic cultures, consisting of proembryonic masses and early cotyledonary somatic embryos that developed on the various induction media, were transferred onto fresh semisolid medium of the same formulation after 2–4 weeks for 1–2 subcultures.

They were thereafter subcultured at 2–4 week intervals on MSP medium (25 ml per 100 x 20 mm Petri dish). The proembryonic masses of 0.1–1 mm diameter were used to form inocula with 0.2–0.5 cm diameter. Up to 7 inocula were plated on each Petri dish. Subculture intervals were 2–4 weeks. The Petri dishes were sealed with Parafilm®.

Effect of medium formulation and gelling agent on the growth and development of 'Thomas' and ' Isham' embryogenic cultures

Factorial experiments consisted of three media formulations and two types of gelling agent. The medium formulations included MS major salts, B5 major salts without $(NH_4)_2SO_4$ ($B5^-$) and $B5^-$ supplemented with 400 mg l^{-1} glutamine; the gelling agents tested were 8 g l^{-1} TC agar and 2 g l^{-1} Gel-Gro® gellan gum (ICN Biochemicals). The major salts formulations were supplemented with MS minor salts, 0.4 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 0.4 μM picloram and 30 g l^{-1} sucrose. The pH of the media was adjusted to 5.7–5.8 with either 0.1–1.0 N KOH or HCl prior to addition of gelling agent. The media were sterilized by autoclaving at 121°C at 1.1 kg cm^{-2} for 15 min. Plant growth media were dispensed in 25 ml aliquots into sterile disposable plastic Petri dishes (100 x 20 mm).

For 'Thomas', the inoculum consisted of 14-day-old embryogenic suspension cultures growing in 80 ml MSP medium in 250 ml Erlenmeyer flasks. Embryogenic cultures were decanted into a sterile funnel layered with 2 layers of sterile Kimwipes tissue paper. Approximately 200–300 mg proembryonic masses were subdivided to form 7 inocula of 0.3–0.5 cm diameter flattened on the surface of the growth medium.

For 'Isham', the inoculum consisted of 14-day-old embryogenic suspension cultures maintained in 80 ml MSP medium in 250 ml Erlenmeyer flasks. The cultures were double sieved through sterile 1.8 mm and 0.8 mm mesh nylon filtration fabric. The smallest proembryonic mass fraction was decanted into a sterile funnel layered with double layers of Kimwipes and was subdivided to form 0.2–0.3 cm diameter inocula on the

surface of the media. There were seven inocula per Petri dish. The Petri dishes were sealed with Parafilm® and maintained in darkness at 25°C.

For 'Thomas', the percent necrotic tissue and the number of somatic embryos that developed per inoculum four weeks after transfer were recorded and analyzed using ANOVA (SAS Institute, 1992). For 'Isham', the numbers of 1) proembryonic masses, 2) globular somatic embryos, 3) hyperhydric and opaque heart stage somatic embryos (diameter <5 mm) and 4) hyperhydric and opaque early torpedo stage somatic embryos (diameter ≥5 mm) per inoculum were recorded after four weeks of culture.

Embryogenic Suspension Cultures

General procedures

The following procedures were applicable for all experiments unless specified otherwise. Medium sterilization was carried out either by autoclaving at 121° C at 1.1 kg cm^{-2} for 15 min or by millipore filter-sterilization with a 0.2 µm sterile filter. For filter-sterilized media, the flasks were sterilized prior to use by autoclaving at 121° C at 1.1 kg cm^{-2} for 20 min. The volume of liquid medium was either 40 ml in 125 ml Erlenmeyer flask (referred to hereafter as 40 ml medium) or 80 ml in 250 ml Erlenmeyer flasks (referred to hereafter as 80 ml medium). The pH of all media was adjusted to 5.7–5.8 with either 0.1–1 N KOH or HCl. The cultures were sealed with heavy duty aluminum-foil and secured with Parafilm®, and maintained in semi darkness at 25°C on a rotary shaker at 125 rpm.

Initiation

Embryogenic suspension cultures were established by inoculating 100–300 mg of 8–10-day-old embryogenic cultures from semisolid MSP medium into either autoclaved or filter-sterilized 40 ml liquid MSP medium.

Maintenance

Embryogenic suspension cultures were subcultured biweekly into filter-sterilized 80 ml MSP medium. For cultures that consisted entirely of proembryonic masses without later stages of development (PEM type), e.g., 'Esther' and 'M25864', 0.8–1.0 g proembryonic masses was used as the inoculum. For cultures up to 12 months old, proembryonic masses were sieved through nylon filtration fabric (1.8 mm or 0.8 mm mesh) and the smallest fractions were used as inocula. For older cultures (>12 months), sieving was unnecessary since the size of proembryonic masses was more homogenous and smaller than in newly established cultures. 'Thomas' cultures were also maintained as described for the PEM type.

For newly established cultures that consisted of proembryonic masses that differentiated as somatic embryos in maintenance medium (SE-type), only proembryonic masses and globular embryos that passed through 0.8 mm mesh nylon filtration fabric were used as inocula. With older cultures that contained dedifferentiated proembryonic masses, 0.8–1 g proembryonic masses that passed through either 1.8 or 0.8 mm mesh nylon filtration fabric were used as the inoculum.

The morphology of embryogenic cultures of different cultivars was observed during a 1–2 year period.

Growth of 'Esther' embryogenic suspension cultures

Embryogenic suspension cultures of 'Esther' were used to determine the growth pattern of embryogenic cultures, because this genotype proliferated in liquid growth medium without concurrent production of early stage cotyledonary somatic embryos. 'Esther' embryogenic suspension cultures (12–14-day-old) maintained in liquid MSP plant growth medium were used as inoculum. Suspension cultures were sieved through sterile nylon 1.8 mm mesh filtration fabric and were retained on 0.8 mm mesh nylon filtration fabric. The sieved cultures that were retained on 0.8 mm mesh filtration fabric were decanted onto a double layer of sterile tissue paper (Kimwipes) and 420 ± 20 mg

proembryos were used as the inoculum in 40 ml autoclaved MSP medium. There were 24 experimental units that consisted of 3 replicates of 8 medium flasks. Each medium flask in a replicate was inoculated with 420 ± 20 mg proembryonic masses that were randomly harvested at day 0, 3, 6, 9, 12, 15, 20 and 25.

Growth parameters that were recorded included the volume of the precipitated culture, fresh weight and dry weight. Precipitated culture volume was measured by decanting cultures into sterile 50 ml graduated centrifuge tubes and allowing them to settle. Fresh weight was determined by pipetting out the liquid medium from the centrifuge tubes and transferring the proembryonic masses to preweighed weighing dishes. Dry weights were obtained by drying the cell masses in an oven at 55° C overnight, and weighing them 30 min after they were removed from the oven. Regression analyses were fitted to the data using Sigma Plot (version 2.0, Jandel™ Scientific, San Rafael, CA).

Effect of picloram on growth of 'M25864' suspension cultures

The effect of picloram on the growth of 'M25864' in suspension culture was examined at six concentrations: 0, 0.13, 0.41, 1.25, 4.14 and 12.48 μM. The plant growth medium used for the experiment consisted of MS salts with 0.1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and picloram as treatment. Each treatment was replicated four times.

The inoculum for the experiment was 14-day-old 'M25864' embryogenic suspension cultures that had been maintained in suspension for ca. one year. Media preparation, preparation of the inoculum and inoculation, cultural conditions and data collection were as described for the growth response experiment above. Regression analyses were fitted to the data using Sigma Plot (version 2.0, Jandel™ Scientific, San Rafael, CA).

Effect of sucrose on the growth of 'M25864' suspension cultures

Sucrose concentration in liquid medium was examined at six different levels: 20, 30, 40, 50, 60 and 70 g l^{-1} . The plant growth medium consisted of MS salts, 0.4 μM picloram, 0.1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol and sucrose as treatment. There were 4 replications per treatment. Medium preparation, source of inoculum, inoculum preparation, inoculation, cultural conditions, data collection and statistical analysis were as described for the picloram experiment.

Effect of thiamine HCl on growth of 'M25864' suspension cultures

The effect of thiamine HCl on the growth of 'M25864' embryogenic suspensions was evaluated at six concentrations: 0, 0.4, 1, 4, 10 and 40 mg l^{-1}. The plant growth medium consisted of MS salts, 0.4 μM picloram, 45 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol and thiamine HCl. Each treatment had four replicates. Media preparation, source of inoculum, inoculum preparation, inoculation, cultural conditions, data collection and statistical analysis were as described for the picloram experiment.

Effect of ascorbic acid and medium sterilization on growth of suspension cultures

Ascorbic acid (100 mg l^{-1}) in the plant growth medium and medium sterilization approach, i.e., autoclaving or filter sterilization, were evaluated in a block factorial design with three replications for two embryogenic lines, 'M25864' and 'Esther'. The plant growth medium used for the experiment was MSP formulation (Table A-1).

The source of inoculum, inoculum preparation, inoculation, cultural conditions and data collection were as described for the picloram experiment. Analysis of variances of the data were computed using Proc. GLM, and t-test was also performed (SAS Institute, 1992).

Results

Induction of Embryogenic Cultures on Semi-solid Medium

Embryogenic cultures were induced from avocado zygotic embryos 18–40 days after explanting and were associated with zygotic embryos of different developmental stages from globular (0.10 mm) to early torpedo stage (2.7 mm). The frequency of somatic embryogenesis of different genotypes on different medium formulations was generally low, from 0 to 25% (Table 3-3). No induction medium effect could be statistically inferred; however, there was an indication that induction medium containing B5 major salts stimulated the greatest embryogenic response from the most genotypes.

Induction of embryogenic cultures generally occurred from the basal part of zygotic embryos, with the single exception of explanted globular zygotic embryos of 'Esther', which were completely embryogenic. It was not clear whether or not the basal part of the zygotic embryos included the hypocotyl region since this basal area was very small. There were two distinct types of embryogenic cultures that were initiated from the explants. 1) Induction of proembryonic masses without heart and later developmental stages of somatic embryos, i.e., PEM type. The PEM type response included 'Esther', and 'M25864'. 2) Induction and development of somatic embryos, including formation of globular to heart stages from the surface of the zygotic embryo explants (Figure 3-1), i.e., SE type. The SE type response included 'Booth 7', 'Booth 8', 'T362', 'Yon', nucellar-derived 'Thomas', zygotic-derived 'Thomas' and 'Isham'.

Induction of embryogenic cultures in nucellar explants occurred primarily from the mycropylar end of the explants (Figure 3-1 C). However, embryogenic cultures were rarely induced from the inner surface of the nucellus. Only two explants of 'Thomas' that were cultured on MSNa and B5 responded out of 18 ovules per treatment. The induction

Table 3-3. Percentage of embryogenic culture induction from immature zygotic embryos from several avocado cultivars on different induction media.

Cultivar	Zygotic Embryo Length (mm)	Rep.	Induction Media[y]				
			MSP	MSNa	MS⁻	MSK	B5
Booth 8	0.1–0.5	10	0 (0–27)[z]	0 (0–27)	0 (0–27)	0 (0–27)	0 (0–27)
	0.6–1.5	10	0 (0–27)	0 (0–27)	0 (0–27)	0 (0–27)	10 (0–40)
	1.6–3.0	10	0 (0–27)	0 (0–27)	20 (3–61)	0 (0–27)	0 (0–27)
Booth 8a	0.8–3.5	11	0 (0–25)	0 (0–25)	9 (3–41)	9 (0.3–41)	18 (2–51)
Esther	0.1–0.5	33	6 (8–20)	0 (0–9)	0 (0–9)	0 (0–9)	0 (0–9)
	0.6–0.8	17	0 (0–9)	0 (0–9)	0 (0–9)	0 (0–9)	0 (0–9)
Isham	0.4–2.0	4	25 (7–80)	0 (0–60)	25 (7–80)	0 (0–60)	0 (0–60)
M25864	1.0–5.0	8	0 (0–30)	0 (0–30)	13 (4–53)	0 (0–30)	13 (4–53)
T362	0.1–0.8	11	0 (0–25)	9 (3–41)	0 (0–25)	0 (0–25)	18 (2–52)
Thomas	0.1–2.0	18	22 (7–48)	0 (0–16)	6 (2–27)	6 (2–27)	4 (7–48)
Yon	0.4–7.0	10	0 (0–27)	20 (3–61)	20 (3–61)	0 (0–27)	10 (0–40)

[y] see Materials & Methods for details

[z] Number in the parentheses indicated binomial confidence interval at 95 %.

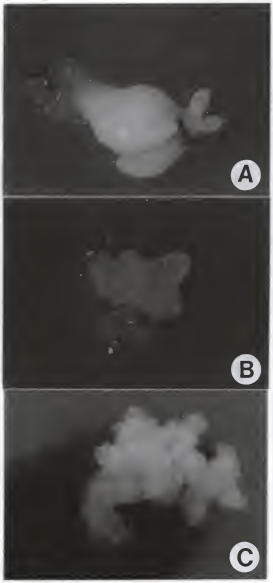
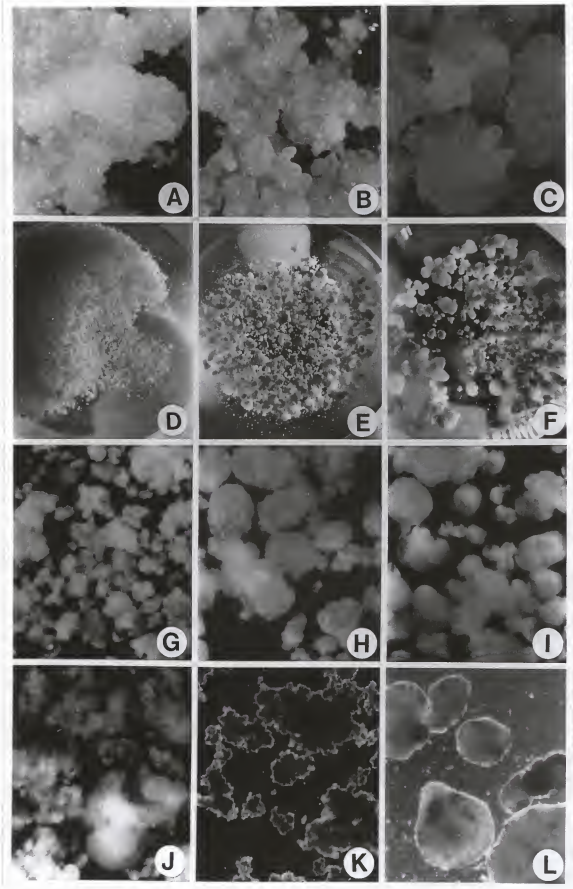


Figure 3-1. Development of secondary somatic embryos from zygotic embryos and proembryonic masses from nucellar explants of 'Thomas' avocado. (A) Secondary somatic embryos developed on the basal region of a cotyledonary zygotic embryo explant of 2 mm length. (B) Similar to (A) except that the zygotic embryo was at heart stage of 0.5 mm length. (C) Proembryonic masses (white) developed from a nucellar explant.

Figure 3-2. Morphological variations of avocado embryogenic cultures. Two distinct type of embryogenesis: PEM-type that is dominated by the appearance of disorganized proembryonic masses (A, D, G and J), SE-type that is dominated by highly organized proembryonic masses and somatic embryos (C, F, I and L), and cultures that are undergoing morphological changes (B, E, H and K). (A) 'Esther' embryogenic cultures on semisolid medium. Note the friable texture and granular nature of the culture. (D) The suspension culture of 'Esther' (A). Note the homogenous nature of the inoculum. (G) Newly established suspension culture of 'Esther' consisting of mostly proembryonic masses and few organized embryogenic masses (arrowhead). (H) 'Esther' embryogenic suspension without organized proembryonic masses. This culture has been in culture longer than (G). (C) 'Isham' embryogenic culture on semisolid medium. Note the development of globular to heart stage somatic embryos covering the entire surface of the proembryonic masses. (F) The development of various stages of somatic embryo development when 'Isham' proembryonic masses were transferred to liquid medium. (I) Another example of SE-type of culture ('Booth 8') which is dominated by development of highly organized proembryonic masses or globular somatic embryo. (B) Embryogenic culture of 'T362' consisting of organized nodular proembryonic masses (lower part) and fine granular and disorganized proembryonic masses. (E) Suspension culture of 'T362'consisted of organized proembryonic masses/somatic embryos, fine granular and disorganized proembryonic masses. (H) A higher magnification of (E). Note the disorganization at the surface of the proembryonic masses. (K) 'Booth 8' suspension culture consisting of disorganized cell clumps incapable of somatic embryogenesis.


A
B
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of nucellar embryogenic cultures for 'Lamb', 'Hass' and 'T362' was also low, i.e. 2%, 0.5% and 6% respectively.

Maintenance of embryogenic cultures on semisolid medium

Transfer of embryogenic cultures (PEM-type) that developed on MSP onto fresh medium of the same composition resulted in continued proliferation of the cultures with occasional development of heart and later stages of somatic embryos. Morphologically, the cultures were friable (Figure 3-2 A) and either yellow-honey ('Esther') or white-brown ('M25864'). After several subcultures, the proembryonic masses were smaller and paler and some of the proembryonic masses had become disorganized. Two to three weeks after routine transfer onto MSP medium, the upper part of the cultures was brown; however, tissue in contact with the medium was healthy and proliferated. Cultures were almost completely necrotic after 5–6 weeks.

Transfer of somatic embryos that developed directly from explanted zygotic embryos (SE-type) to medium of the same composition, e.g., B5, MSK, MSNa, resulted in maturation of the somatic embryos and formation of a few secondary somatic embryos from the basal part of the somatic embryos. They were alternatively subcultured onto MSP medium. After several transfers onto MSP medium, proliferation of proembryos, proembryonic masses and globular somatic embryos was enhanced (Figure 3-2 B). Proembryos were characterized by granular morphology and small size (ca. 0.1–0.2 mm). The globular somatic embryos were characterized by granular morphology with smooth surfaces and ca. 0.3–1 mm diameter and the formation of secondary somatic embryos at one pole instead of scattered around the entire surface. The proembryonic masses were characterized by their nodular morphology with diameter of ca. 1–2 mm and the formation of secondary somatic embryos and proembryonic masses on their surfaces. These structures gave rise to a mixture of globular, heart and cotyledonary stage somatic embryos and proembryonic masses ca. 2–3 weeks after transfer. The somatic embryos

that developed in this medium were mostly hyperhydric and their shapes were distorted. Early and late heart stage somatic embryos produced proembryonic masses and secondary globular somatic embryos from their bases. Therefore the gross morphology of this type of culture (SE-type) was dominated by the appearance of proembryonic masses and hyperhydric somatic embryos at various stages of development.

SE-type cultures were maintained by subculturing proembryos, proembryonic masses and globular somatic embryos. After ca. 5–10 subcultures, depending on the genotype, the embryogenic cultures also consisted of smaller cell masses with less organization, and which appeared to be disorganized proembryonic masses. In one genotype, 'Isham', disorganized proembryonic masses did not develop. Instead, globular, heart and cotyledonary stage somatic embryos developed from the entire surface of proembryonic masses (Figure 3-2 C). Distal to the culture medium, the cultures became brown 2–3 weeks after transfer.

Effect of medium composition and gelling agent on growth and development of nucellus-derived 'Thomas' and 'Isham' embryogenic cultures.

Medium composition significantly affected the percentage of 'Thomas' cultures that became necrotic after four weeks. MS formulation resulted in the lowest frequency of necrosis (Table 3-4). Type of gelling agent had no significant effect on necrosis of cultures nor was there an interaction of gelling agent with medium with respect to necrosis. Somatic embryo development was not significantly affected by the different treatments.

Table 3-5 demonstrated that the proliferation of 'Isham' proembryonic masses was significantly affected by media formulation and the interaction of media formulation with gelling agent. The highest value of proembryonic mass proliferation occurred with MS major salt formulation and solidified with TC agar (Figure 3-3).

Table 3-4. Effect of major salt composition, and gelling agent on necrosis and somatic embryogenesis of 'Thomas' nucellar culture.

Major salts[a]	Gelling agent	Necrotic tissue (%)	No. somatic embryos[b]
		(Mean ± SE)	(Mean ± SE)
MS	8 g l^{-1} TC Agar	17 ± 8	0.28 ± 0.15
	2 g l^{-1} Gel-Gro	27 ± 16	0.46 ± 0.28
B5	8 g l^{-1} TC Agar	100 ± 0	0.74 ± 0.21
	2 g l^{-1} Gel-Gro	100 ± 0	0.51 ± 0.16
B5'G	8 g l^{-1} TC Agar	100 ± 0	0.57 ± 0.23
	2 g l^{-1} Gel-Gro	100 ± 0	0.00 ± 0.00
Anova Summary			
Major salt (M)		**[c]	NS[d]
Gelling agent (G)		NS	NS
M*G		NS	NS

[a] Major salt of MS (Murashige & Skoog, 1962), B5 (Gamborg et al., 1968), B5'G is B5 major salts without $(NH_4)_2 SO_4$, with 400 mg l^{-1} glutamine.

[b] Number of early stage somatic embryos per inoculum.

[c] Significant at $\alpha = 1\%$; [d] Not Significant at $\alpha = 1\%$

Table 3-5. Summary of the value of Pr>F from ANOVA for the effect of major salt composition, and gelling agent on the proliferation of proembryonic masses and development of somatic embryos from 'Isham' cultured on semisolid medium.

Dependent Variable		Component		
		Major Salts (M)	Gelling Agent (G)	M*G
		..... Pr>F		
Proembryonic masses[1]		0.0060	0.1188	0.0037
Globular SE[1]		0.2620	0.7634	0.0101
Cotyledonary SE<5mm	Opaque[2]	0.0001	0.1176	0.0574
	Hyperhydrous[2]	0.0041	0.0840	0.0051
	Total[1]	0.0002	0.0001	0.0400
Cotyledonary SE≥5 mm	Opaque[2]	0.0544	0.7294	0.1126
	Hyperhydrous[2]	0.0001	0.0001	0.0001
	Total[1]	0.0002	0.0001	0.0400
Total Hyperhydrous SE[2]		0.0001	0.0001	0.0157
Total Opaque SE[1]		0.0008	0.2811	0.0493
Total Cotyledonary SE[1]		0.0017	0.0004	0.0561

[1] data were normally distributed, therefore no transformation.

[2] data were not normally distributed and were transformed with arc sin transformation.

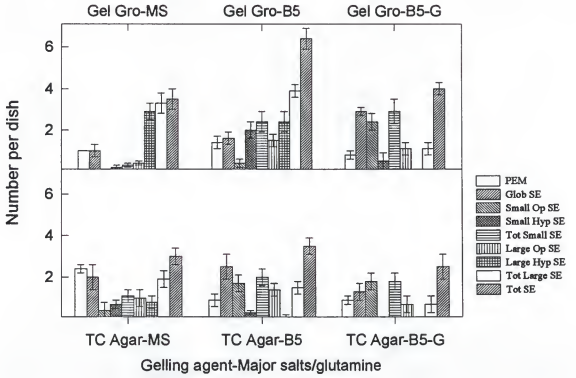

Figure 3-3. Effect of gelling agent, major salts and glutamine on the proliferation of proembryonic masses and somatic embryo development of 'Isham' avocado.

Somatic embryo growth and development, as indicated by different sizes, stages and hyperhydricity, was prolific with 'Isham'. The development of globular somatic embryos was not significantly affected by medium and gelling agent but was significantly affected by their interaction. Medium with B5-G solidified with Gel-Gro™ resulted in the highest number of globular somatic embryos. Opaque cotyledonary somatic embryo < 5 mm diameter development was only affected by medium, with the highest value occurring on medium B5-G. The presence of hyperhydric cotyledonary somatic embryos < 5 mm was significantly affected by medium and the interaction of medium with gelling agent, and the highest number occurred with the treatment of B5 and Gel-Gro™. The total number of cotyledonary somatic embryos < 5 mm was significantly affected by medium formulation, gelling agent and their interaction, and the highest number was obtained with medium consisting of B5-G and Gel-Gro™. There was no significant effect of treatment on development of opaque cotyledonary somatic embryos ≥ 5 mm diameter. Hyperhydric cotyledonary somatic embryos ≥ 5 mm diameter were significantly affected by media formulation, gelling agent and their interactions, with the highest number obtained on MS with Gel-Gro™. Total number of cotyledonary somatic embryos ≥ 5 mm was significantly affected by media formulation ($P>F = 0.0002$) and gelling agent ($P > F = 0.001$) but not by their interaction ($P>F = 0.04$). Duncan Multiple Range Test (DMRT) indicated that B5 medium was optimum for total somatic embryos followed insignificantly by MS and significantly by B5-G. T-test indicated that Gel-Gro™ resulted in more somatic embryos than TC agar ($P> T = 0.0075$). The effect of treatment on number of somatic embryos (of all stages) was the same as the effect on the total number of large somatic embryos.

Initiation of Embryogenic Suspension Cultures.

Rapidly growing, 8–10 day-old proembryonic masses with a uniform white or yellow color were used as inoculum. All genotypes, with the exception of 'Isham', that

were established successfully on semisolid medium could also be established in liquid MSP medium.

Maintenance

The two distinct types of cultures, PEM-type and SE-type, described on semisolid medium, retained these characteristics in newly-established embryogenic avocado suspensions. In the PEM-types, i.e., 'Esther' and 'M25864', the suspensions consisted of less organized proembryonic masses of different sizes with a low frequency of globular and heart stage somatic embryos (Figure 3-2 D, G). In the SE type, the suspensions consisted primarily of globular to cotyledonary somatic embryos (Figure 3-2 F, L). The PEM-type could be maintained by subculturing any fraction of the cultures; however, maintenance of the SE-type required the smallest fraction of cultures (<0.8 mm mesh size) as inoculum. Newly established cultures of both types of suspensions were characterized by production of abundant cell debris.

With time and regular subculture, the morphology of embryogenic suspension cultures changed. Globular, heart and early cotyledonary stage somatic embryos that occurred at low frequency in the PEM-type, e.g., 'Esther', started to disappear after 12 months and the cultures consisted of increasingly disorganized proembryonic masses (Figure 3-2 J).

Similar morphological changes also occurred in SE-type cultures, with several variations. For example, proembryonic masses or globular somatic embryos derived from zygotic embryos of 'T362' and 'Lamb' lost their ability to organize as heart stage somatic embryos, but retained their morphology 6–18 months before becoming completely disorganized. The periphery of proembryonic masses and globular somatic embryos underwent limited disorganization (Figure 3-2 H), which eventually resulted in loss of organization (nucellar-derived 'T362', 'Booth 8', 'Yon'). Globular somatic embryos

developed from proembryonic masses, but partially dedifferentiated, forming less organized proembryonic masses (nucellar-derived 'Thomas', 'Hass') or formed partially disorganized proembryonic masses and large, free vacuolate cells (zygotic embryo-derived 'Thomas', 'Hass') before completely disorganizing. Completely disorganized proembryonic masses consisted of small isodiametric cells in clusters that were either slow growing ('Lamb') or fast growing ('T362', zygotic embryo-derived 'Thomas') (Figure 3-2 K).

'Isham' could not be maintained in suspension. The proembryonic masses that were inoculated developed into globular to cotyledonary stage somatic embryos in the presence of picloram. Therefore 'Isham' was maintained by alternating subculture of semisolid with liquid media; proembryonic masses from semisolid medium were inoculated into liquid medium and globular somatic embryos and proembryonic masses in liquid medium were subcultured onto semisolid medium before they could develop as later stage somatic embryos. Unorganized proembryonic masses were never recovered from cultures of 'Isham'; however, the size of somatic embryos that developed on semisolid or liquid medium decreased with time.

Time required for organized proembryonic masses or globular somatic embryos to become completely disorganized was genotype-dependent (Table 3-6).

Growth responses of 'Esther' Embryogenic Suspension Cultures

'Esther' embryogenic suspension culture growth pattern could be fitted with a curvelinear trend with respect to tissue volume, fresh weight and dry weight. The fitted regression lines had high coefficient of correlation values (r^2 = 0.96–0.98) (Figure 3-4). There was a short lag phase (ca. 1 day) with respect to volume of 'Esther' embryogenic suspension cultures, followed by 5 days of exponential growth, 9 days of linear phase, 5 days of progressive decelerating growth and stationary or declining growth through 25

Table 3-6. Characteristics of avocado embryogenic suspension cultures.

Cultivar of Mother Tree	Explant	Embryogenesis Type	Time Required for PEM Disorganization
Booth 7	zygotic	SE	8–12 month
Booth 8	zygotic	SE	8–12 months
Esther	zygotic	PEM	12–24 months
Hass	nucellus	SE	6–12 months
Isham	zygotic	SE	never
Lamb	nucellus	SE	3–6 months
M25864	zygotic	PEM	8–12 months
T362	zygotic	SE	12–18 months
T362	nucellus	SE	6–12 months
Thomas	zygotic	SE	12–18 months
Thomas	nucellus	SE	12–18 months
Yon	zygotic	SE	3–6 months

days of culture. A trend similar to volume variable was observed for fresh weight, except that the lag phase was ca 1 day longer and the exponential phase was 1 day shorter. Dry weight, however, increased without an apparent lag phase, but with a sharp exponential phase for ca 4 days, followed by a linear phase for 8 days, and progressively decelerating phase for 6 days and declining thereafter. The peak for each variable was predicted by its regression line to occur at different days. Dry weight peaked at 18 days, followed by fresh weight (20 days) and volume (21 days). Increases in volume, fresh weight and dry weight under the growth conditions described were ca 14-fold, 6.4-fold and 7.9-fold, respectively.

During the declining phase, loss of dry weight from the peak to the end of culture period accounted for ca. 40%. This dry weight loss was much higher than the loss in volume and fresh weight, each of which accounted for 17%.

Effect of picloram, sucrose and thiamine HCl on the growth of 'M25864' embryogenic suspension cultures.

Regression analysis of volume and dry weight with respect to picloram concentration resulted in very low r^2 values (< 0.05), indicating that picloram had no effect on those growth variables. The fresh weight of the cultures decreased curvelinearly (r^2 = 0.37) with picloram concentration (Figure 3-5). Therefore, the concentration of 0.41 µM (0.1 mg l^{-1}) was retained as the standard concentration of picloram for suspension cultures.

Sucrose affected the volume, fresh and dry weight of 'M25864' embryogenic suspension cultures curvelinearly; however, its coefficient of correlation was low (r^2 = 0.34–0.35). The volume and fresh weight of the embryogenic suspension culture peaked at a sucrose concentration of ca. 25 mg l^{-1}, while the dry weight peaked at ca. 50 g l^{-1}. The increase in dry weight that resulted from increased sucrose concentration from 20 to 50 g l^{-1} may indicate increased starch content since it coincided with the decrease of volume and fresh weight of cultures at a sucrose concentration of 25 g l^{-1} and thereafter. Sucrose concentration of 30 g l^{-1} has been used as standard sucrose concentration in the medium.

Regression analysis of the effect of thiamine HCl concentration in the medium demonstrated that r^2 for volume, fresh weight and dry weight of the culture were very low, i. e. 0.16, 0.11 and 0.004, respectively. This low value indicated that thiamine HCl was not very critical for growth. Nevertheless, based on the increasing trend of the volume and fresh weight with increasing thiamine HCl concentration from 0 to 10 mg l^{-1}, a thiamine HCl concentration of 4 mg l^{-1} has been adopted as standard for avocado maintenance medium (MSP).

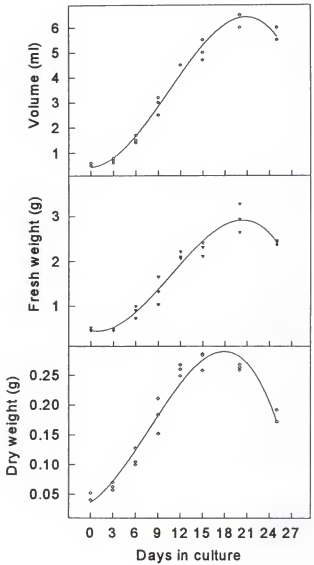

Figure 3-4. Growth response of 'Esther' avocado embryogenic suspension cultures over time (T). The regression lines are represented by A) volume = 0.44 +0.013 T + 0.040 T2 - 0.0013 T3, r2 = 0.98, B) fresh weight = 0.46 - 0.03 T + 0.021 T2 - 0.00067 T3, r2 = 0.96 and C) dry weight = 0.03 +0.0081 T + 0.0015 T2 - 0.000063 T3, r2 = 0.96.

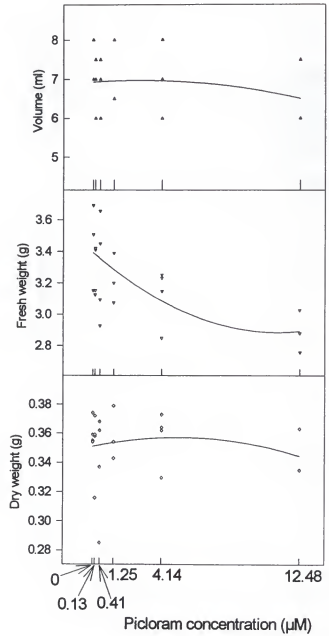

Figure 3-5. Effect of picloram (P) on growth response of 'M25864' avocado embryogenic suspension cultures after 14 days. The growth responses and their regression lines are represented by: (a) volume, $Y = 6.9 - 0.03\ P - 0.005\ P^2$, $r^2 = 0.05$; (b) $Y = 3.4 - 0.1\ P - 0.004\ P^2$, $r^2 = 0.37$; (c) $Y = 0.35 - 0.02\ P - 0.0002\ P^2$, $r^2 = 0.03$.

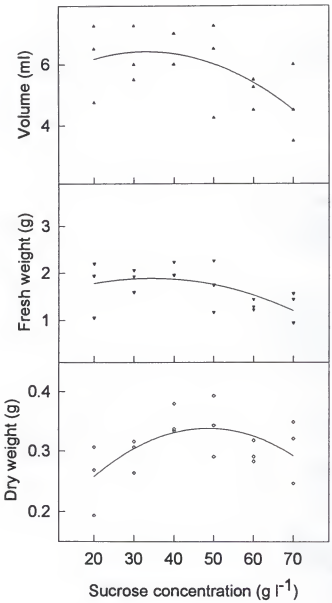

Figure 3-6. Effect of sucrose (S) on the growth response of 'M25864' avocado embryogenic suspension cultures after 14 days. The growth responses and their regression lines are represented by: (a) volume, $Y = 4.9 - 0.09\ S - 0.0014\ S^2$, $r^2 = 0.35$; (b) $Y = 1.25 - 0.037\ S - 0.0005\ S^2$, $r^2 = 0.34$; (c) $Y = 0.1 - 0.01\ S - 0.0001\ S^2$, $r^2 = 0.35$.

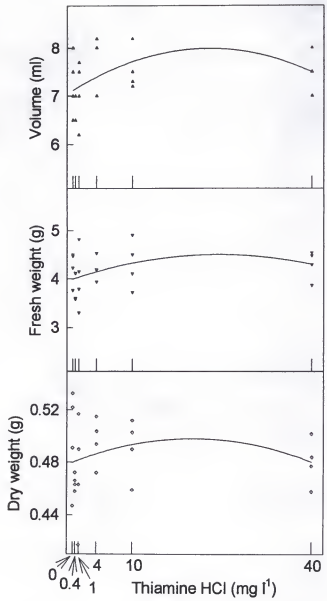

Figure 3-7. Effect of thiamine HCl (T) on the growth response of 'M25864' avocado embryogenic suspension culture after 14 days. The growth responses and their regression lines re represented by: (a) volume, $Y = 7.1 - 0.07\ T - 0.002\ T^2$, $r^2 = 0.15$; (b) $Y = 4.0 - 0.04\ T - 0.008\ T^2$, $r^2 = 0.11$; (c) $Y = 0.47 - 0.02\ T - 0.0005\ T^2$, $r^2 = 0.004$.

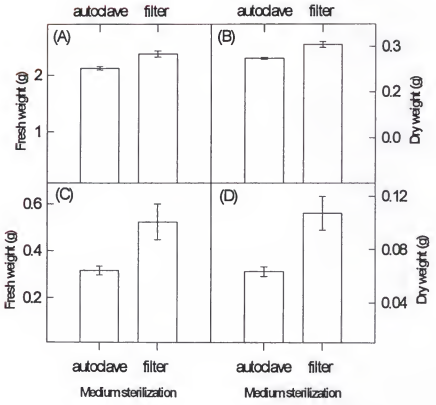

Figure 3-8. Effect of medium sterilization protocol on the growth of avocado embryogenic suspension cultures. Data from cultures in medium with and without ascorbic acid were pooled to assess effects of medium sterilization protocol. Medium was either autoclaved at 1.1 kg cm^{-2}, 121° C for 15 min or filtered with 0.2 µm filter unit. (A) Fresh weight of 'Esther' avocado, (B) dry weight of 'Esther' avocado, (C) fresh weight of 'M25864' avocado, (D) dry weight of 'M25864' avocado. Bars in the histograms represent standard errors of the means.

Effect of ascorbic acid and medium sterilization method on growth of suspension cultures

Ascorbic acid in the medium and its interaction with the medium sterilization method did not significantly affect ($P > 0.05$) the growth of either 'Esther' or 'M25864' embryogenic suspension cultures. Medium sterilization method significantly affected fresh weight ($P < 0.01$) and dry weight ($P < 0.01$) of 'Esther', and fresh weight ($P < 0.05$) and dry weight ($P < 0.05$) of 'M25864'embryogenic suspension cultures. Data from cultures in plant growth media with or without ascorbic acid were pooled to determine affects of medium sterilization protocol. The t-test showed that filter sterilization resulted in significantly higher fresh weight ($P < 0.01$ for 'Esther'; $P < 0.05$ for 'M25864)' and dry weight ($P < 0.01$ for 'Esther'; $P < 0.05$ for 'M25864') than autoclaving (Figure 3-8). Therefore, filter sterilization has been used as the standard protocol for preparing liquid maintenance medium.

Discussion

Somatic embryogenesis in avocado had been reported previously using immature zygotic embryos as explants (Skene & Barlass, 1983; Mooney & Van Staden, 1987; Pliego-Alfaro & Murashige, 1988). Using a similar method, embryogenic cultures have been established both on semisolid and in liquid medium from immature zygotic embryos and from nucellar explants obtained from 12 avocado cultivars/genotypes.

The establishment of avocado embryogenic cultures was characterized by direct formation of proembryonic masses or somatic embryos from the explant without apparent development of callus. This embryogenic culture establishment has been referred to as the proembryogenic determined cell (PEDC) pathway (Sharp et al.,1980) or permissive induction pathway (Ammirato, 1987), in contrast to the induced embryogenic determined cell (IEDC) pathway (Sharp et al., 1980) or directive induction pathway (Ammirato, 1987), which involves the formation of callus prior to the development of somatic embryos. The embryogenic response of avocado nucellar and zygotic embryo explants

was quite low. Low frequency of embryogenic culture initiation from immature zygotic embryos or nucellar explants has been reported for some other woody species, e.g., <1% for big leaf magnolia (Merkle & Warson-Pauley, 1993), <5% for *Cornus floribunda* (Trigiano et al., 1989) and pecan (Corte-Olivares et al., 1990). A low embryogenic response was also observed from the nucellus of monoembryonic *Citrus* spp., e.g., microcitrus (Vardi et al., 1986) and seedless 'Cohen' citrange (Grosser et al., 1993). However, a high embryogenic response (60–80%) was reported from immature zygotic embryo explants of *Quercus robus* (Chalupa, 1995). Moore (1985) found that ovules of monoembryonic *Citrus* spp. did not proiduce embryogenic cultures while the polyembryonic species produced somatic embryos. Chaturvedi and Mitra (1972) indicated that a correlation appeared to exist between degree of polyembryony and somatic embryogenesis.

The size of zygotic embryo explants was reported to be critical for initiating avocado embryogenic cultures (Pliego Alfaro & Bergh, 1992; Mooney & van Staden, 1987); however this variable may be genotype-dependent, since embryogenic cultures could be induced from zygotic embryos ca. 2 mm length in the current study. Nucellar tissues from very young fruit could also be used for initiating embryogenic cultures at low frequency. Induction of embryogenic avocado cultures from nucellar explants could have significant horticultural implications. Somatic embryogenesis from avocado nucellus could replace the expensive etiolation technique that is currently used for clonally propagating avocado rootstock cultivars (Frolich & Platt, 1971). Single character improvement using elite scion genotypes could also be accomplished via genetic transformation.

Establishing and maintaining embryogenic avocado cultures was based upon initiation of repetitive or secondary somatic embryogenesis from cultures on/in plant growth medium containing B5 major salts and then using the secondary somatic embryos as explants on medium containing MS major salts. B5 major salts-containing medium with

a low concentration of reduced inorganic nitrogen appears to favor somatic embryo development and has been used for mango somatic embryo development (Litz et al., 1991; 1995; Mathews & Litz, 1992). Muralidharan & Mascarenhas (1995) also demonstrated that *Eucalyptus citriodora* embryogenic masses proliferated in MS-based medium, while somatic embryo development (maturation) increased on B5-based medium.

Another characteristic of embryogenic avocado cultures was the distinction between the PEM-type (2 embryogenic culture lines) and the SE-type (12 embryogenic culture lines) that was evident after culture initiation and which was consistent during maintenance on semisolid medium and in liquid medium. This distinction could be an important marker for predicting somatic embryogenesis frequency since the PEM-type is associated with lower frequency of development of somatic embryos (see Chapter 4). Proliferation of proembryonic masses requires an exogenous auxin and its withdrawal results in somatic embryo development (Litz & Gray, 1995). Therefore, the different types of avocado embryogenic cultures reflect differential sensitivity to auxin. The different types of somatic embryogenesis in avocado may also indicate differences in the integrated development of the somatic proembryos (William & Maheswaran, 1986).

Embryogenic cultures resembling avocado of the PEM-type, i.e., characterized by discreet globular or clusters of globular, organized structures, yellow or white, with gross morphology consisting of friable, granular/nodular structures, have also been reported for *Salix viminalis* (Grönros, 1995), *Rosa hybrida* (Robert et al., 1995), cacao (Figuera & Janick, 1995), apple (Wallin et al., 1995) and cotton (Finer, 1988). The histology of an avocado embryogenic culture has been reported by Mooney & Van Staden (1987) to consist of somatic proembryos from 0.1–1.0 mm. The nodular structures were actually proembryonic masses, since they lacked a discernible protoderm and proembryos continually formed on the surface of the proembryonic masses. These structures resemble proembryonic masses that constitute embryogenic cultures of citrus (Button et al., 1974) and mango (DeWald, 1987; Litz et al., 1995; 1992, etc.). Ammirato (1984) indicated that

when proembryos are maintained in induction medium, they may not organize as somatic embryos but will continue to enlarge and form proembryonic masses that form secondary proembryos on their surface.

Avocado embryogenic cultures can be maintained both in liquid suspension cultures and on solid medium. The ease of proliferation and maintenance of avocado embryogenic cultures would provide regenerable materials for further culture manipulation involving somatic cell genetics or transformation. Using zygotic-derived embryogenic culture of 'Thomas', Cruz-Hernandez et al. (1998) reported *Agrobacterium*-mediated transformation of avocado with GUS and NPT II genes and recovery of transformed somatic embryos.

Changes involving gross morphology of embryogenic cultures of the SE-type occurred after a relatively short time and can be implicated in the progressive loss of embryogenic potential of these cultures. William and Maheswaran (1986) suggested that the loss of integrated organization of globular embryos is associated with appearance of proembryonic masses in embryogenic cultures. The morphology of disorganized avocado proembryonic masses is similar to embryogenic cultures that have become "undifferentiated" and which have been selected from the most friable nucellar *Citrus deliciosa* cultures (Cabasson et al., 1995) and from habituated nucellar cultures of *Citrus aurantium* L. (Gavish et al., 1991) even though these cultures could be induced to form somatic embryos. Chaturvedi and Mitra (1975) found that after prolonged subculture the organized globular embryogenic culture initiated from stems of *Citrus grandis* became highly friable, but embryogenic.

Embryogenic avocado suspension cultures of the PEM-type grow as typical suspension cultures, with a 2 week subculture period with a 5.5-fold increase in mass, etc. Most of the attempts to improve growth of PEM-type embryogenic cultures in liquid medium by altering the organic addenda of the plant growth medium were not successful. Increase in dry weight of the suspension cultures with 50 g l^{-1} sucrose was demonstrated

but 30 g l^{-1} sucrose has been used routinely. Increased dry weight with sucrose concentrations above 30 g l^{-1} only resulted in accumulation of starch in the cells. High starch content in embryogenic cells must be avoided if cultures are to be used as a source for protoplasts (Grosser, 1994). Filter sterilizing liquid medium significantly improved the growth of avocado embryogenic cultures. Medium autoclaving has been reported to cause the formation of toxic substance as the result of breakdown of sucrose and other carbohydrates (Shiao & Bornman, 1991).

The loss of embryogenic potential of avocado embryogenic cultures in a relatively short time could be disadvantageous since 1) studies of somatic embryo development, maturation and germination require several months (see Chapter 4) so that the same cultures cannot be used indefinitely; 2) gene transformation or somaclonal variation experiments that require long periods of subculturing (selection) may result in loss of embryogenic potential and failure to recover somatic embryos. Therefore conservation methods are required to solve this problem. Preliminary experiments have indicated that avocado embryogenic cultures can be initiated from embryogenic cultures on semisolid medium that have been stored for 6 months at 6–10° C. However, cryopreservation might be explored for this species, using proembryonic masses, microcalli and early globular stage somatic embryos. Cryopreservation protocols and subsequent plant regeneration have been developed for different tropical plant trees, (Withers, 1992; Engelman, 1991), including nucellar-derived embryogenic cultures (Kobayashi et al., 1990; Sakai et al., 1990), proembryonic masses and early stage somatic embryos (Marín et al., 1993) of navel orange (*Citrus sinensis* (L.) Osb.), embryogenic cells of rice (*Oryza sativa* L) (Jain et al., 1996), embryogenic cell suspensions and callus cultures of cotton (*Gossypium hirsutum* L.) (Rajasekaran, 1996), and sweet potato (*Ipomoea batatas* (L.) Lam) (Blakesley et al., 1996).

In conclusion, embryogenic cultures of avocado have been established on semisolid and maintained on semisolid or in liquid medium from several genotypes (including four

elite selections) with diverse genotypic background from either zygotic embryos or nucellar explants. Establishment of suspension cultures has been especially critical for further studies involving somatic cell genetics, protoplast isolation, culture and somatic hybridization and plant transformation. The loss of embryogenic potential of cultures after a few months may pose problems related to lack of homogeneity of experimental cultures over time. Conservation techniques, i.e., cryopreservation, may circumvent this problem.

CHAPTER 4
SOMATIC EMBRYO DEVELOPMENT, MATURATION AND GERMINATION

Introduction

Pliego-Alfaro & Murashige (1988) reported that avocado somatic embryos were hard, white, glistening and egg shaped. The development of these somatic embryos from embryogenic cultures occurred in low frequency and was not affected by sucrose concentration, ABA, casein hydrolysate and activated charcoal (Pliego-Alfaro & Murashige, 1988).

In Chapter 3, the establishment of avocado embryogenic cultures and their maintenance as suspension cultures were reported. The embryogenic cultures were characterized by the presence of proembryonic masses and various stages of hyperhydric somatic embryos on both semisolid proliferation medium and in suspension culture. Efficient incipient somatic embryo development of several embryogenic avocado genotypes in suspension culture has been demonstrated (Chapter 3); however, these somatic embryos failed to develop further. In addition, the ability of cultures to form somatic embryos in liquid medium diminishes with age of the culture.

In this chapter, efficient production of good quality somatic embryos by manipulating some of the physical conditions of culture is described.

Materials and Methods

General Procedures

Plant materials used in these experiments consisted of proembryonic masses derived from suspension cultures that were grown in 80 ml filter sterilized MSP medium (Table A-1) in 250 ml Erlenmeyer flasks maintained at 120 rpm in semidarkness for 14 days. Embryogenic cultures on semisolid medium were maintained in darkness. All cultures were maintained at 25°C.

Effect of Embryogenic Suspension Culture-derived Somatic Embryo Stage and Size on the Development of Somatic Embryos on Semisolid Medium

'Isham' embryogenic suspension cultures were used as the source of inoculum. 'Isham' embryogenic cultures consisted of proembryonic masses and somatic embryos of different sizes at early stages of development. These cultures had been subcultured 2–3 times after inoculation with proembryonic masses that had been maintained on semisolid MSP (Table A-1) medium for over six months (2–4 week subculture period in liquid medium).

The treatments consisted of three different sizes and developmental stages of somatic embryos that were used as the inoculum: 1) proembryonic masses with diameter <0.8 mm with or without globular to early heart stage somatic embryos attached; 2) heart stage somatic embryos with a cotyledon of 0.8–1.5 mm width and 0.3–0.5 cm length; 3) cotyledonary stage somatic embryos with cotyledons >2 mm width and >0.5 cm length. The inocula were cultured on SED medium (Table A-1). There were nine inocula per Petri dish (110 x 20 mm) and there were five Petri dishes per treatment.

Data were subjected to analysis of variances to determine the treatment effect (SAS Institute, 1992). Treatment means were separated using standard errors and they were presented graphically.

Effect of Gelling Agent Concentration on the Development of Somatic Embryos

The effect of different gelling agent concentrations in the medium was evaluated with Gel-Gro gellan gum at 2, 3, 4, 5, 6, 7, 8, 9, 10 g l^{-1} and TC agar at 8 g l^{-1} as the control. The basal medium was SED medium (Table A-1) with the gelling agent as treatment.

The source of inoculum was 14-day-old 'T362' embryogenic suspension cultures that had been maintained for >8 months with a 2-week subculture interval. 'T362' embryogenic suspension cultures consisted of different sizes of proembryonic masses and proembryonic mass clumps, but with no somatic embryo development. The inoculum for the experiment consisted of 5–10 mg proembryonic masses that passed through sterile nylon filtration fabric of 1.8 mm mesh and were retained on sterile nylon filtration fabric of 0.8 mm mesh. There were 9 inocula per Petri dish (110 x 20 mm) and there were 4 replications Petri dishes per treatment. The number of hyperhydric and opaque somatic embryos and their sizes were recorded after 1 month of culture.

Analysis of variance was computed to determine the treatment effect. Treatment means were separated using standard errors and they were presented graphically.

Effect of Sucrose Concentration and the Size of Proembryonic Masses on the Development of Opaque Somatic Embryos on Semisolid Medium

A factorial experiment was carried out to determine the effects of sucrose concentration in the plant growth medium and size of proembryonic mass inoculum on the development of opaque somatic embryos grown on semisolid medium. The sucrose concentrations tested were 10, 30, 50, 70, 90, 110 and 130 g l^{-1}, and the size of proembryonic masses ranged from 0.8 to 1.8 mm diameter (passed through 1.8 mm mesh nylon filtration fabric and retained on 0.8 mm mesh nylon filtration fabric) and >1.8 mm diameter (retained on 1.8 mm mesh nylon filtration fabric). The plant growth medium was SED formulation (Table A-1) with sucrose concentration as treatments.

The source of inoculum consisted of 'T362' embryogenic suspension cultures as described previously. There were 9 inocula (0.05-0.12 g each) per replication and there were 4 replicates per treatment.

Data were subjected to analysis of variances to determine the treatment effect (SAS Institute, 1992). Treatment means were separated using standard errors and they were presented graphically.

Effect of Carbon Source on the Growth and Development of Cultures that Have Lost Embryogenic Competence

Galactose and glycerol at concentration of 5.4% (w/v) and 2.8% (v/v), respectively, in addition to a control (3% sucrose) were tested for their effect on somatic embryo development in liquid medium. The inoculum was derived from nucellar 'Thomas' cultures that had been maintained for over one year. The culture consisted of dedifferentiating proembryonic masses (350–1500 µm diameter). The inoculum, consisting of 200 mg proembryonic masses, was inoculated in 40 ml liquid medium in 125 ml Erlenmeyer flasks. The medium composition was SED (Table A-1) with carbon source as the treatment.

Effect of Total Nitrogen Concentration and Ratio of NO_3^-:NH_4^+ on the Growth and Development of 'T362' Avocado Embryogenic Cultures

The effects of total inorganic nitrogen concentration (30 mM and 60 mM) and the ratio of NO_3^-/NH_4^+ (1:0, 3:1, 1:1, 1:3 and 0:1) on the growth and development of embryogenic cultures that consisted of disorganized proembryonic masses were evaluated in suspension in a factorial experiment. The NO_3^-:NH_4^+ ratio was formulated using KNO_3, NH_4NO_3 and $(NH_4)_2SO_4$ according to Niedz (1994). The composition of the medium was that of SED medium (Table A-1) with inorganic nitrogen according to treatments. The

inoculum consisted of 0.4 g dedifferentiating proembryonic masses of 'T362' in 40 ml liquid medium. There were 5 replicates per treatment.

Fresh weight and pH of the cultures were observed after 2 weeks in culture. Analysis of variances were performed to determine interaction (SAS Institute, 1992). Regression analyses were fit to the data using Sigma Plot™ (Jandel Scientific, San Raphael, CA). For regression analyses, the NO_3^-:NH_4^+ ratios were expressed as NO_3^- percentage from total inorganic nitrogen and set as the x axis.

Somatic Embryo Maturation and Germination

Opaque cotyledonary somatic embryos (> 0.8 cm diameter) that developed on SED medium were transferred individually onto semisolid Somatic Embryo Maturation and Germination (SEMG) medium (Table A-1). Aliquots of 25 ml medium were dispensed into 150 x 25 mm glass test tubes, closed with polypropylene Kaputs, autoclaved for 15 min at 121°C and 1.1 kg cm^{-2} and cooled as slants.

After subculture of each somatic embryo, the tubes were closed with Suncaps™ and secured with rubber bands. The cultures were kept upright in darkness at 25°C. The somatic embryos were subcultured onto fresh medium of the same composition at 2-3 month intervals. When root or shoot growth was apparent, the cultures were transferred to 16 h light provided by cool white fluorescent tubes (80–100 $\mu mol\ s^{-1}\ m^{-2}$).

Shoot Proliferation and Plantlet Regeneration from Shoot-Derived Somatic Embryos

The shoot that emerged from each somatic embryo that developed from a 'T362'-derived embryogenic culture was decapitated 1-1.5 cm from the tip, cultured on Shoot Multiplication (SM) medium (Table A-1) according to Witjaksono (1991) and subcultured at 8 week intervals for several passages. They were thereafter subcultured on

Avocado Shoot Proliferation (ASP) medium (Table A-1). Rooting of shoots has been described in Chapter 6 and follows the protocol of Pliego-Alfaro (1988).

Results

Subculturing proembryonic masses or globular somatic embryos on medium similar to proliferation medium without plant growth regulators resulted in low frequency occurrence of opaque, white somatic embryos that resembled zygotic embryos. On medium without plant growth regulators, the somatic embryos were generally hyperhydrous and distorted. Under optimal conditions, healthy opaque, white mature somatic embryos developed through globular, early and late heart, cotyledonary stages as indicated in Figure 4-1 A-G. These somatic embryos occasionally developed shoots without roots. The shoots could be propagated and rooted (Figure 4-1 H). The optimal conditions for this development were determined as follows.

Effect of Embryogenic Suspension Culture-derived Somatic Embryo Stage and Size on Development of Somatic Embryos on Semisolid Medium

This experiment was carried out in order to determine if developmental stage of somatic embryos that developed in liquid medium affected their further development on semisolid medium. Late heart stage somatic embryos that developed in liquid medium failed to develop further on semisolid medium. Their size remained the same and their color became dull and opaque; however, smooth, opaque secondary somatic embryos developed from the plated somatic embryo inocula. Development of proembryonic masses inoculated from liquid onto semisolid medium proceeded differently. Some of the proembryonic masses died; however, secondary somatic embryogenesis occurred from some of the cultures. The secondary somatic embryos were different in size, and they were a mixture of hyperhydric and opaque embryos. Since only opaque and large somatic

Figure 4-1. Avocado somatic embryo development from proembryonic masses and subsequent plant regeneration. (A-E) Developmental stages of 'Booth 7' avocado somatic embryos from globular to cotyledonary. Note that in C, the two cotyledons were apart while in the zygotic counterpart, those cotyledons remain close to each other (see Table 3-2). (F) 'Booth 7' somatic embryos, mostly at cotyledonary stage, enlarged to 0.8-1.2 cm diameter after two months on SED medium. (G) Multiple shoot development from a 'Booth 7' somatic embryo ca. one year after its initiation. (H) A plantlet and a proliferating shoot that developed from a somatic embryo. The somatic embryo developed from zygotic embryo-derived 'T362' avocado.

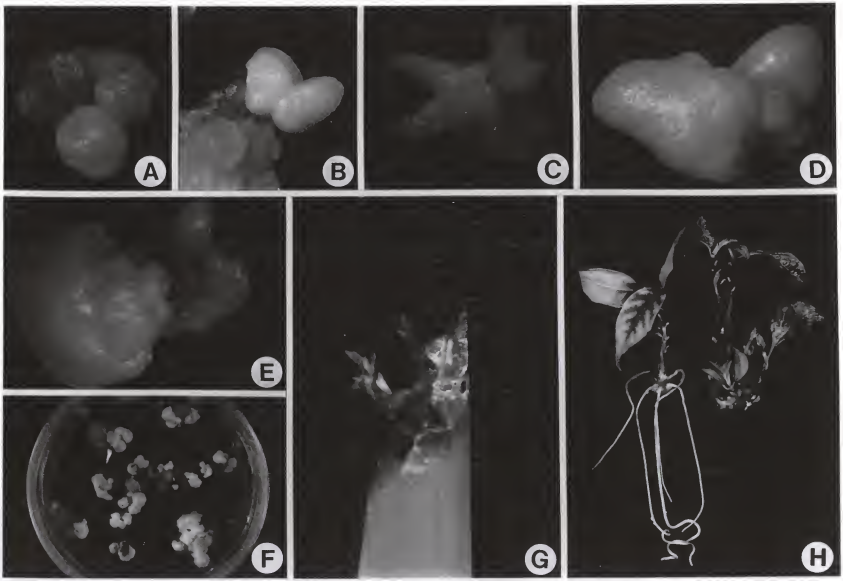
A
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Table 4.1. ANOVA of the effect of 'Isham' embryogenic suspension culture-derived somatic embryo size and stage of development on development of secondary somatic embryos on semisolid medium.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Number of secondary opaque somatic embryos ≥0.8 cm diameter					
Treatment	2	525.733	262.867	66.83	0.0001
Error	12	47.200	3.933		
Total	14	572.933			
Number of somatic embryos that developed root					
Treatment	2	1.733	0.8667	2.60	0.1153
Error	12	4.000	0.333		
Total	14	5.733			

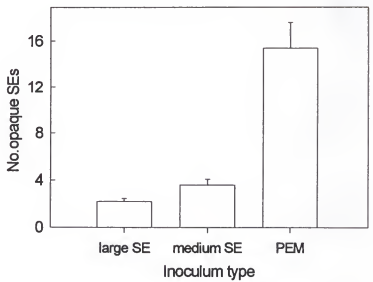

Figure 4-2. Opaque cotyledonary stage somatic embryo production as affected by inoculum type derived from 'Isham' avocado liquid embryogenic cultures after one month on semisolid medium. Large SE = cotyledonary somatic embryos with cotyledon size of > 2 mm width and > 5 mm length; Medium SE = cotyledonary somatic embryos with cotyledon size of > 0.8–1.5 mm width and > 3–5 mm length; PEM = proembryonic masses with diameter < 0.8 mm with or without globular to heart stage somatic embryos attached.

embryos (>0.8 cm) matured normally, only the appearance of this type of somatic embryo was recorded.

Table 4.1 indicated that inoculum size and developmental stages of somatic embryos used as the inoculum significantly affected the number of opaque somatic embryos produced per dish, but had no effect on root emergence of the somatic embryos. The largest number of opaque cotyledonary somatic embryos per dish was obtained when the proembryonic masses were used as the inoculum; and the lowest number was obtained when the largest somatic embryos were used as the inoculum (Figure 4-2). Somatic embryo development in this avocado embryogenic genotype was very efficient.

Effect of Gelling Agent Concentration on Development of Somatic Embryos

After 1 month of culture, the growth and development of the cultures were visually distinguishable among treatments. On treatments consisting of 8 g l^{-1} TC Agar and 2 g l^{-1} Gel-Gro™ there was no somatic embryo development, although loose nodular and granular proembryonic masses and distorted heart to torpedo stage hyperhydric somatic embryos proliferated. With increasing Gel-Gro™ concentration, somatic embryos with well formed cotyledons were observed. While there was no necrotic tissue in the treatment 2 g l^{-1} Gel-Gro™ and 8 g l^{-1} TC Agar, the percentage of necrotic tissue that developed from the distal part of the culture increased with Gel-Gro™ concentration.

Analysis of variance (Table 4-2) indicated that Gel-Gro™ concentration significantly affected the number of opaque, hyperhydric somatic embryos, the total number of somatic embryos and the size of somatic embryo (width and length) that developed on semisolid medium.

Figure 4-3 demonstrated that the length of somatic embryos increased slightly on medium with 3 to 5 g l^{-1} Gel-Gro™ and then decreased with a Gel-Gro™ concentration of

10 g l^{-1}. A significant decrease in somatic embryo length occurred with Gel-Gro™ at 7 g l^{-1}. The number of hyperhydric somatic embryos increased slightly, while the number of

Table 4-2. ANOVA of effect of Gel-Gro™ concentration on development of opaque and hyperhydric somatic embryos and size of opaque somatic embryo from 'T362' proembryonic masses cultured on semisolid medium.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Number of opaque somatic embryos					
Treatment	8	69.60	8.70	12.21	0.0001
Error	27	19.23	0.71		
Total	35	88.83			
Number of hyperhydrous somatic embryo					
Treatment	8	121.32	15.16	13.02	0.0001
Error	27	31.45	1.16		
Total	35	152.77			
Total number of somatic embryo					
Treatment	8	277.85	34.73	23.44	0.0001
Error	27	40.00	1.48		
Total	35				
Width of somatic embryo					
Treatment	8	19.61	2.45	19.54	0.0001
Error	27	3.39	0.13		
Total	35	22.97			
Length of somatic embryo					
Treatment	8	39.84	4.98	22.06	0.0001
Error	27	6.09	0.23		
Total	35	45.94			

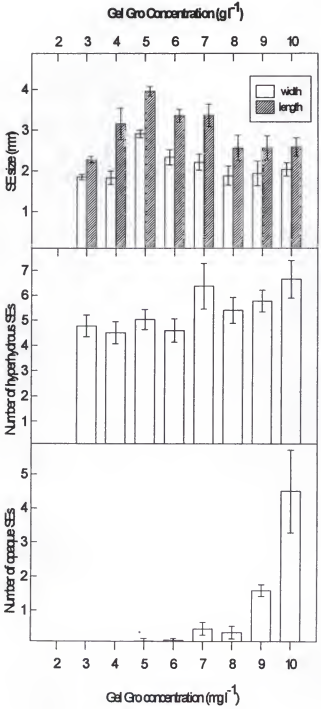

Figure 4-3. Effect of Gel-Gro™ concentration on development of somatic embryos from proembryonic masses on semisolid medium after one month of culture.

opaque somatic embryos increased sharply with increasing Gel-Gro™ concentration. Good quality opaque somatic embryos were obtained with 6 g l^{-1} Gel-Gro™. At 6 g l^{-1} Gel-Gro™, 0.12 opaque somatic embryos developed from each inoculum. Since there were 9 inocula per Petri dish, 1–2 opaque cotyledonary somatic embryos could be subcultured for somatic embryo germination. At 7 g l^{-1} Gel-Gro™, 5–7 opaque somatic embryos could be recovered. Since no significant difference in somatic embryo size was detectable between 6 and 7 g l^{-1} Gel-Gro™, these Gel-Gro™ concentrations were used as standards.

With Gel-Gro™ concentrations of 6–7 g l^{-1}, good quality cotyledonary somatic embryo development has been obtained for cultures that were initiated from zygotic embryos of 'Booth 7' (Figure 4-1 F), 'Booth 8', 'Yon' and 'Thomas'. Somatic embryo development was also obtained from embryogenic suspensions of 'M25864' that could not form somatic embryos in liquid medium. Somatic embryo development on this Gel-Gro™-modified medium was also obtained with 'Hass', 'Thomas' and 'Lamb' cultures that were initiated from nucellar explants. Somatic embryo development was not observed on this medium from cultures that were derived from 'Esther' zygotic embryos.

Effect of Sucrose Concentration and the Size of Proembryonic Masses on the Development of Opaque Somatic Embryos on Semisolid Medium

The development of opaque somatic embryos of various stages of development (from heart to cotyledonary) was affected neither by size of proembryonic mass inocula nor its interaction with sucrose concentration, but was significantly affected by sucrose concentration (Table 4-3). No opaque somatic embryos developed at sucrose concentrations of 10 g l^{-1}. Opaque somatic embryos developed only at sucrose concentrations of at least 30 g l^{-1}. The number of opaque somatic embryos increased with a sucrose concentration of 90 g l^{-1} and then decreased with sucrose concentrations up to 130 g l^{-1} after 1 month in culture (Figure 4-4).

Table 4-3. ANOVA of the effect of sucrose concentration and the size of proembryonic masses on production of opaque somatic embryos of various stages of development.[z]

Source	DF	Sum of Squares	Mean Squares	F Value	Pr > F
Sucrose	6	4296.66	716.11	25.33	0.0001
Size	1	59.62	59.62	2.11	0.1539
Sucrose*Size	6	383.58	63.93	2.26	0.0557
Error	42	1187.58	28.27		
Total	55	5927.43			

[z] from suspension culture-derived proembryonic masses of 'T362' on semisolid medium.

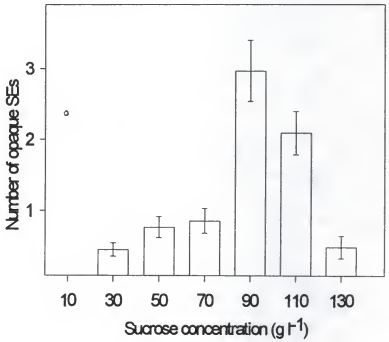

Figure 4-4. Effect of sucrose concentration on the production of opaque somatic embryos on semisolid medium.

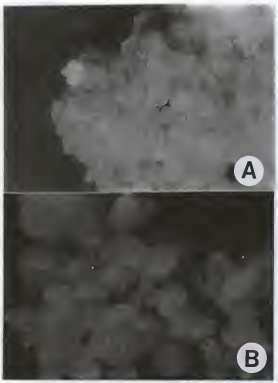

Figure 4-5. Somatic embryo development as affected by high sucrose concentration. (A) Globular to early heart stage somatic embryos developed on the entire surface of zygotic-derived 'T3623' cultured on SED with 13% sucrose 3 months after culture (B) Development of late heart to cotyledonary stage somatic embryos one month after transfer from 13% sucrose to 3% sucrose containing SED medium.

With increasing sucrose concentration, smaller and earlier stages of somatic embryos developed; at a concentration of 90 g l^{-1} there were only globular and heart stage somatic embryos. Therefore, the sucrose concentration of 30 g l^{-1} was retained as the standard. The proembryonic masses on media with high sucrose concentrations (90–130 g l^{-1}) become compact and deep yellow in color, and there was a low frequency of early stage somatic embryo (globular to heart) development on their surface. After 3 months, globular somatic embryos covered the entire inoculum (Figure 4-5 A). Transfer of these cultures onto SED medium with 30 g l^{-1} sucrose and 6 g l^{-1} Gel-Gro™ resulted in development of clusters of somatic embryos from heart to early cotyledonary stages (Figure 4-5 B).

Effect of Carbon Source on the Growth and Development of 'Thomas' Cultures that Had Lost their Embryogenic Potential

Microscopic observation revealed that cultures maintained in liquid medium with sucrose consisted of 100–200 μm diameter granular proembryonic masses, 1–2 mm diameter nodular proembryonic masses, dedifferentiated proembryonic masses and a low frequency of free cells. In medium with galactose as the carbon source, the cultures appeared to be similar, except that nodular proembryonic masses of 1–2 mm diameter had lost their integrative appearance. In medium with glycerol, free round and elongated cells were observed more frequently than in cultures with other carbon sources. Nevertheless sucrose, galactose and glycerol had no effect on restoration of embryogenic potential of these cultures.

Different carbon sources significantly affected the growth and proliferation of the cultures as indicated by their final volume and fresh weight after 2 weeks in culture. There was no significant difference between galactose and sucrose with respect to culture fresh weight; however, galactose and sucrose resulted in significantly higher fresh weight than

glycerol. Volume of the cultures was greatest in medium with galactose and smallest in medium with glycerol (Figure 4-6).

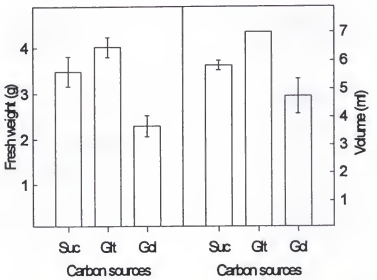

Figure 4-6. Effects of carbon source on fresh weight and volume of embryogenic nucellar 'Thomas' avocado cultures. The inoculum consisted of mixed, nodular and dedifferentiating proembryonic masses. Inoculum of 0.4 g was inoculated in 40 ml liquid medium. Data were taken after two weeks in culture. Medium composition was MS without PGR with carbon source as treatment: 3% (w/v) sucrose (Suc), 5.4% (w/v) galactose (Glt) and 2.8% (v/v) glycerol (Gcl).

The effect of total nitrogen concentration and ratio of NO_3^-:NH_4^+ on the growth and development of 'T362' embryogenic cultures

After 2 weeks, there were differences in color and morphology of the cultures (Table 4-4). Globular and early cotyledonary stage somatic embryos developed in three treatments, i.e., 60 mM nitrogen consisting of 75 and 100% NO_3^- and 30 mM nitrogen consisting of 100% NO_3^-. Only dedifferentiating proembryonic masses were present in the other treatments.

Table 4-4. The effect of total N and $\%NO_3^-$ on color and morphology of avocado 'T362' suspension cultures.[z]

N total (mM)	NO_3^- percentage	Color of culture	SE	PEMs	
				Nodular	Dedifferent-iating
60	100	dark yellow	+	+	+
	75	yellow	+	-	+
	50	whitish yellow	-	-	+
	25	whitish brown	-	-	+
	0	whitish brown	-	-	+
30	100	yellow	+	+	+
	75	pale yellow	-	-	+
	50	whitish yellow	-	-	+
	25	whitish brown	-	-	+
	0	whitish brown	-	-	+

[z] Inoculum was taken from dedifferentiating proembryonic masses that had been in culture for over 1 year. + = present, - = absent

There was a significant interaction between nitrogen level and $\%\ NO_3^-$ with respect to fresh weight gain (Table 4-5). Regression analyses indicated that fresh weight gain responded curvelinearly with $\%\ NO_3^-$ (Figure 4-9). Final medium pH was significantly affected by $\%\ NO_3^-$ but not by total nitrogen concentration and interaction between $\%\ NO_3^-$ and total nitrogen concentration (Table 4-5). Therefore, data for total nitrogen concentration were pooled for regression analysis against $\%\ NO_3^-$. Final medium pH increased quadratically with increasing $\%\ NO_3^-$ (Figure 4-7). At 0–30% NO_3^-, the medium pH fell to <4.0, and then increased linearly with increasing $\%\ NO_3^-$ to 100%.

At total nitrogen concentration of 60mM, fresh weight gain increased with increasing $\%\ NO_3^-$ from 0 to 75% and then decreased with $\%\ NO_3^-$ though 100%. This curve can be divided into 2 response groups: low response, which was a function of $\%\ NO_3^-$ from 0 to 50%, and high response, which was a function of $\%\ NO_3^-$ from 50 to 100%. In the low response, fresh weight gain ranged from 1 to 7 fold, while in the high

response, fresh weight gain ranged from 7 to 11 fold. The high response group coincided with high pH, while the low response group coincided with low pH. The fresh weight gain peaked at ca. 82.5% NO_3^- at ca. pH 5.7.

A similar pattern occurred when the total nitrogen of the medium was lower, e.g., 30 mM; however, the fresh weight responses against % NO_3^- were generally lower than with 60 mM. At 30 mM nitrogen, the high response group corresponded with % NO_3^- from 70–100. This interval also coincided with high final medium pH. At this level of nitrogen, fresh weight increase peaked at ca. 85%, NO_3^- at pH 6.5.

Table 4-5. ANOVA for the effect of nitrogen concentration and % NO_3^- on proembryonic mass fresh weight gain and medium pH in liquid medium.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Fresh weight increase					
Treatment	9	5.7.47	56.38	38.24	0.0001
Error	33	48.65	1.47		
Total	42	556.13			
Per cent NO_3^+	4	420.42	105.10	71.29	0.0001
N concentration	1	47.17	47.17	31.99	0.0001
Percent*Concentration	4	36.62	9.15	6.21	0.0008
Medium pH					
Treatment	9	72.96	8.10	64.08	0.0001
Error	34	4.30	0.12		
Total	43	77.26			
Per cent NO_3^+	4	70.59	17.64	139.51	0.0001
N concentration	1	0.17	0.17	1.34	0.2547
Percent*Concentration	4	1.74	0.44	3.45	0.0181

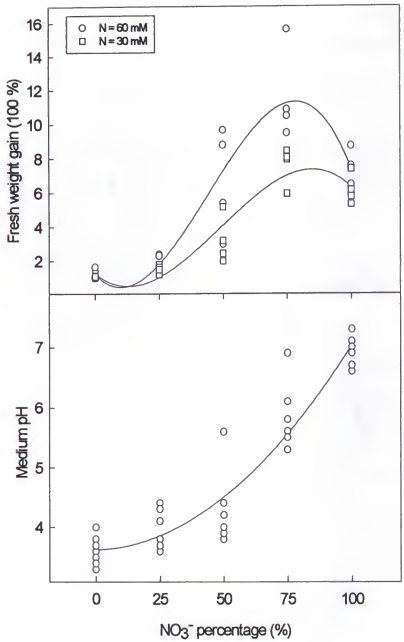

Figure 4-7. Effect of total nitrogen concentration and NO_3^-/NH_4^+ ratio on culture fresh weight gain (x 100%) and medium pH. Ratio of NO_3^-/NH_4^+ are expressed as NO_3^- percentage and set as the X axis. The fitted regression lines were represented as follows: fresh weight gain at 60 mM N, $Y_1 = -10.7 + 27.3\ x - 10.3\ x^2 + 1.1\ x^3$, $R^2 = 0.84$; fresh weight gain at 30 mM nitrogen, $Y_2 = -1.4 + 12.4\ x - 5.2\ x^2 + 0.57\ x^3$, $R^2 = 0.88$; medium pH, $Y_3 = 3.6 + 0.0009\ x + 0.0003\ x^2$, $R^2 = 0.91$.

Somatic Embryo Maturation and Germination

Upon transfer to somatic embryo maturation medium, cotyledonary somatic embryos with diameter > 8 mm continued to enlarge to ca. 1-2 cm diameter. Proembryonic masses were present in some of the cultures. After ca. 9-10 months, 2.5% (1/40), 5% (3/60), 5% (2/40) of 'T362', 'Booth 8' and 'Booth 7' somatic embryos, respectively, developed shoots. No shoot development was observed from about 40 somatic embryos of 'M25864' and 200 somatic embryos of 'Isham'. Upon transfer to light, the somatic embryos turned green, and the shoots elongated (Figure 4-1 G).

Shoot Proliferation and Plantlet Regeneration from Shoot-Derived Somatic Embryos

Only a single shoot grew from the single axillary bud on each nodal explant and from the apical bud of each shoot tip. Upon transfer to ASP medium, multiplication rates of 6 fold for each 8-week subculture cycle were obtained. The *in vitro* shoots could be rooted with a frequency of 60%. Rooting generally occurred after 2-4 months in culture. Figure 4-1 H represents proliferating shoots and rooted shoots of somatic embryo origin.

Discussion

In most of the embryogenic genotypes, avocado somatic embryos developed in liquid medium in the presence of plant growth regulators. These somatic embryos, however, have never been able to develop to maturity in liquid medium as they become necrotic and died. Those somatic embryos also failed to develop further and died on semisolid medium; however, secondary somatic embryos that grew from them developed normally. The development of somatic embryos to maturity occurred with high frequency when proembryonic masses were used as inoculum instead of later stage (cotyledonary) somatic embryos. Somatic embryos of certain mango cultivars, on the other hand, can

initiate development in liquid medium and continue to develop on semisolid medium (DeWald et al., 1989b).

It was possible to control the development of avocado somatic embryos by manipulating certain physical parameters, i.e., gelling agent and sucrose concentration, in addition to the genotype effect. Gelling agent affected somatic embryo development in avocado in two ways: 1) a specific level is required for differentiation and 2) a higher level is required for development of opaque instead of hyperhydric somatic embryos. Increasing gelling agent has been reported to reduce hyperhydricity even though it decreases other growth parameters in shoot cultures of *Cynara scolymus* (Debergh et al., 1981), *Gerbera jamesonii, Forsythia intermedia, Oreopanax nymphaeifolium* (Debergh, 1983) and *Amelachier arborea* (Brand, 1993). Increasing gellan gum concentration from 2 to 6 g l^{-1} also increased the reversion of hyperhydric to opaque somatic embryos in mango (Monsalud et al., 1995). Increasing gellan gum concentration increases the gel rigidity/medium hardness (Huang et al., 1995), lowers medium matrix potential (Debergh et al., 1981, Debergh, 1983), and lowers medium water potential (Ghashghaie et al., 1991).

Sucrose can affect somatic embryo development in two ways: 1) as a carbon source and 2) as an osmoticum which affects morphogenesis (DeWald et al., 1989b). As a carbon source, as indicated by its effect on biomass accumulation, sucrose is optimum for growth of embryogenic avocado suspensions at concentration of 30-50 g l^{-1} (see Chapter 3). The higher sucrose concentration may function as osmoticum that controls morphogenesis (Litz & Conover, 1982). The effect of sucrose on avocado somatic embryo development may be genotype-dependent since 11-13% sucrose resulted in development of globular somatic embryos from proembryonic masses of 'T362' but not from 'Booth 8' and 'Yon' (data not shown). Pliego-Alfaro & Murashige (1988) reported that the percentages of cultures with somatic embryo development from embryogenic

cultures derived from 'Hass' zygotic embryo explants was not significantly affected by sucrose concentration.

On somatic embryo development medium that is solidified with 6–7 g l^{-1} gellan gum, avocado somatic embryos developed from proembryonic masses as globular or nodular stage, heart stage and later to a mature stage. Only somatic embryos that were opaque and white could enlarge further, developed shoots and turned green under light condition.

The morphology of embryogenic suspension cultures changed with time from globular, nodular proembryonic masses and somatic embryos to dedifferentiating proembryonic masses without apparent somatic embryo development, indicating diminishing (or even loss of) embryogenic potential (see Chapter 3). Somatic embryo development could not occur from cultures that had lost their embryogenic potential (unpublished results). By replacing sucrose in the medium with galactose (Kochba et al., 1978; Kochba et al., 1982; Cabasson et al., 1995), lactose (Kochba et al., 1978; Kochba et al., 1982), raffinose (Kochba et al., 1978) and glycerol (Ben-Hayyim & Neuman, 1983), somatic embryo development could be increased dramatically from embryogenic cultures of citrus. Somatic embryo development could also be recovered from citrus embryogenic cultures that had apparently lost embryogenic potential in sucrose-containing medium by replacing sucrose with galactose (Cabasson et al., 1995), or glycerol (Gavish et al., 1991; Vu et al., 1993). Attempts to obtain somatic embryos from similar avocado cultures by replacing sucrose with galactose or glycerol were unsuccessful. Differential growth of avocado suspension cultures in plant growth media with 3 different carbon sources has also been reported in citrus. In avocado suspensions, galactose medium resulted in greater fresh weight and volume of sedimented cultures, although growth parameters of citrus suspensions were lower with galactose compared to sucrose-containing medium (Cabasson et al., 1995; Kochba et al., 1978). The lower growth rate of avocado suspension cultures in medium containing glycerol compared to those in medium

containing sucrose is in agreement with results for citrus (Gavish et al., 1991; Vu et al., 1993).

Varying the salt composition, especially the nitrogen content of the plant growth medium, affected somatic embryo development from embryogenic cultures (see Chapter 3). Niedz (1994) reported that the NO_3^-:NH_4^+ ratio affected only the growth of citrus embryogenic cultures, but had no effect on either embryogenic potential or culture morphology. In contrast, the ratio of NO_3^-:NH_4^+ affected both growth and differentiation of an avocado embryogenic culture that had lost its embryogenic potential.

Avocado cultures were able to grow in liquid medium with KNO_3 as the sole nitrogen source, but were unable to grow in liquid medium with only NH_4Cl. Optimum growth occurred when both NO_3^- and NH_4^+ were present in the medium in a ratio of ca. 3:1. The ability of plant cells to grow in medium with KNO_3 as the only nitrogen source has been reported, even though better growth was obtained when both NO_3^- and NH_4^+ were present in the medium (Kirby et al., 1987). The low rate or absence of growth of avocado suspension in medium with low nitrate content (low NO_3^-:NH_4^+ ratios) may be due to NH_4^+ toxicity. NH_4Cl as the sole nitrogen in the medium was inhibitory to *Pinus strobus* callus growth due to NH_4^+ and not Cl^- (Kaul & Hoffman, 1993). The NH_4^+ toxic effect could be alleviated by incorporating KNO_3 in the medium (Kaul & Hoffman, 1993). Despite their susceptibility to ammonium ion toxicity, plant cells can be adapted to grow in NH_4Cl as the sole nitrogen source when acids of the tricarboxylic acid cycle are provided (Kirby et al., 1987)

The importance of the type of nitrogen (reduced or oxidized form) for somatic embryogenesis has been classically demonstrated by Halperin & Wetherell (1968). The association of efficient somatic embryo development with high NO_3^-:NH_4^+ ratios has been suggested previously for avocado on semisolid medium that consisted of B5 salts compared to MS (see Chapter 3). Similar results have been reported for mango, in which B5 medium stimulated higher somatic embryo development than MS or ½ MS (DeWald et

al., 1988) and for a forest tree *Ocotea catharinensis* (Moura-Costa et al., 1993). Using dedifferentiating proembryonic masses, avocado somatic embryos could be recovered in medium with high NO_3^-:NH_4^+ ratio while no somatic embryos could be recovered in medium with low NO_3^-:NH_4^+ ratio. The somatic embryos that developed in liquid medium with a high ratio of NO_3^-:NH_4^+ (1:0 to 3:1), however, failed to enlarge. Maturing avocado somatic embryos were routinely obtained on MS medium with a NO_3^-:NH_4^+ ratio of 2:1 (see Chapter 4; Pliego-Alfaro & Murashige, 1988; Mooney & Van Staden, 1987). This may indicate a differential requirement of nitrogen ratio, which is related to the stage of development of the somatic embryos. Joy et al. (1996) observed that different amino acids accumulated in somatic embryos of different stages of development. Different amino acids in the medium also encouraged the growth of carrot somatic embryos of different stages of development (Higashi et al., 1996).

Avocado somatic embryo development to germination requires ca. 9–10 months. This time span corresponds to the time required from fruit set to fruit maturity, which is 6–12 months, depending on the cultivar (Whiley, 1992). At maturity, avocado seeds are large, 3–7 cm in diameter, whereas mature avocado somatic embryos are only 1.5–2.0 cm diameter. The plant conversion rate from avocado somatic embryos has been low, ca. 0–5%, and similar to that reported by Pliego-Alfaro and Murashige (1987) and Mooney and Van Staden (1988).

The failure of shoot development has been attributed to failure of shoot meristem development in maturing carrot somatic embryos (Nickle & Yeung, 1993). Similar observations were also found from histological studies of somatic embryos of avocados (Pliego-Alfaro & Murashige, 1988; Mooney & Van Staden, 1987). Those histological studies demonstrated that even though there were meristematic regions in the basal and apical region of the somatic embryos, they were much less pronounced than the zygotic embryo counterpart. In carrot, such failure could be corrected by incorporating ABA in the medium (Nickle & Yeung, 1993). In contrast, Pliego-Alfaro & Murashige (1988)

found that ABA, cold treatment or GA_3 had no effect on shoot development from avocado somatic embryos. Fujiwara and Komamine (1975) found that cytokinin was important for organization of the apical meristem of carrot somatic embryos. Attempts to improve plant conversion by incorporating BA in the maturation medium were unsuccessful (data not shown). The failure of treatments to overcome this developmental anomaly may be because they were applied too late during development. Barlass and Skene (1983) reported that immature avocado zygotic embryos developed shoots with greater frequency when they were taken from abscised fruit >6-weeks old. Apparently the first 6-7 weeks of embryo development is a critical period for somatic embryo development, and somatic embryos of this stage should be pulsed with a shoot-inducing treatment to improve plant conversion. Alternatively, maturation treatments such as culturing somatic embryos on medium with high osmoticum, slow desiccation, ABA treatment or their combination (Etienne et al., 1993; Pence, 1992; Pliego-Alfaro et al., 1995a; b) should also be tried to increase plant conversion. Shoots that develop from somatic embryos, however, could be micropropagated, and plantlets could be regenerated using standard protocols.

CHAPTER 5
PROTOPLAST ISOLATION, CULTURE AND SOMATIC EMBRYO REGENERATION OF AVOCADO

Introduction

Avocado production worldwide has been threatened by root rot disease caused by an Oomycete, *Phytophthora cinnamomi* Rands, especially in areas with heavy soil and poor drainage (Zentmeyer, 1980; Zentmeyer et al., 1994). Crop production in affected areas has been dependent on the use of a few tolerant roostocks (Zentmeyer et al., 1994). Conventional breeding for resistance to root-rot disease has been impeded due to low heritability of the tolerance trait (Coffey, 1987), long juvenility period and low fruit set (ca. 0.001%) (Blanke & Lovatt, 1995), all of which are typical obstacles in breeding perennial fruit crops.

Protoplast culture and regeneration-based biotechnology approaches have been demonstrated to be very important tools for complementing conventional breeding (Gmitter et al., 1992; Ochatt et al., 1992). For example, with citrus, following the first report of somatic hybridization between 'Trovita' sweet orange *Citrus sinensis* and *Poncirus trifoliata* (Ohgawara et al., 1985), this technique has been successfully utilized to produce somatic hybrid plants from at least 150 parental combinations (Grosser, 1993; Ohgawara et al., 1994; Grosser et al., 1994; Mourao-Filho, 1995; Louzada & Grosser, 1994; Ling & Iwamasa, 1995; J. W. Grosser, personal communication) that have been intended for various purposes, i.e., production of allotetraploid hybrids for rootstocks, for crosses with diploids to produce seedless scion cultivars and introgression of useful traits from citrus relatives that are sexually incompatible and for confirming the hybrid origin of certain species. Production of citrus cybrid plants has been achieved through asymmetric

donor-recipient protoplast fusion (Vardi et al., 1987), electrofusion (Saito et al., 1993) and PEG-mediated fusion of protoplasts (Grosser et al., 1996) and could eventually allow the study of cytoplasmic inheritance of certain characters. Direct gene transfer to citrus protoplasts and recovery of transgenic plants has also been described (Kobayashi & Uchimiya, 1989; Vardi et al., 1990; Schell, 1991).

Citrus somatic hybridization has been based on the availability of protoplast-to-tree protocols for several species, e.g., *C. sinensis* (Kobayashi et al., 1985; Grosser & Gmitter, 1990; Vardi & Galun, 1988), *C. mitis* (Sim et al., 1988), *C. aurantium, C. limon, C. paradisi* and *C. reticulata* (Vardi & Galun, 1988). In addition, protocols for protoplast culture and regeneration for citrus relatives have been reported for *Microcitrus* (Vardi et al., 1986), *Murraya paniculata* (Jumin & Nito, 1995), *Atalantia bilocularis, Hesperethusa crenulata, Glycosmis pentaphylla, Triphasia trifolia, Murraya koenigii* (Jumin & Nito, 1996a) and *Citrosis schweinfurthii* (Jumin & Nito, 1996b).

Reports of protoplast culture and regeneration of other fruit trees have been limited to deciduous woody perennials in the Rosaceae family, e.g., *Malus* spp., *Prunus* spp. and *Pyrus* spp. (Ochatt, 1990; Ochatt, 1993; Ochatt et al. 1992), including a haploid 'Golden Delicious' apple clone (*Malus* X *domestica* Borkh.) (Patat-Ochatt et al., 1993), 'Starkrimson' apple (Ding et al., 1995), sour cherry (*Prunus cerasus* L.) (Ochatt, 1990), colt cherry (*Prunus avium* X *pseudocerasus*) (Ochatt, 1990) and pear (*Pyrus communis* L.) (Ochatt & Power, 1988). Protoplast culture and regeneration has also been reported for woody perennial fruit vines in the family Vitaceae, i.e., *Vitis vinifera* (Kovalenko & Galkin, 1990), *Vitis* sp. (Reustle et al., 1995) and in the family Actinidiaceae, i.e., *Actinidia deliciosa* var. *deliciosa* 'Hayward' (Oliveira & Pais, 1991), and among some tropical and subtropical fruit species including *Passiflora edulis* (Manders et al., 1991) and *Diospyros kaki* (Tao et al., 1991). Somaclonal variants that showed high levels of salt and drought tolerance were recovered from protoplast-derived cultures of *Prunus avium* X *pseudocerasus* (Ochatt and Power, 1989) and somaclonal variants of *Pyrus communis*

that showed differences in rootability have been reported (Ochatt, 1987). Somatic hybrid plants have been obtained following protoplast fusion only between 'Colt' cherry (*Prunus avium)* and *Pyrus communis* (Ochatt et al, 1989) despite attempts with other combinations in or between these genera (Ochatt & Patat-Ochatt, 1994) and between *Passiflora* spp. (Dornelas et al., 1995; Otoni et al., 1995).

Application of protoplast-based technologies to avocado improvement could have great utility, particularly with respect to breeding for root-rot resistance. Protoplast fusion could be used to overcome sexual and graft incompatibility barriers between avocado and the root-rot resistant small-seeded *Persea* species in the subgenus *Eriodaphne* (Pliego-Alfaro & Bergh, 1992; Bergh & Lahav, 1996), e.g., *P. borbonia, P. cinerascens, P. pachypoda, P. caerulea,* etc. (Zentmeyer, 1980). Production of transgenic rootstocks could be accomplished by direct transfer of antifungal genes, i.e., β-glucanase and/or chitinase (Lamb et al., 1992), into protoplasts of existing rootstocks.

There have been only been a few reports concerning either protoplast culture or regeneration of avocado. Protoplasts have been isolated from nonmorphogenic avocado callus for studying sunblotch viroid replication (Blickle et al., 1986) and from fruit mesocarp tissue for study of fruit ripening (Percival et al., 1991); however, regeneration from protoplasts derived from these tissues was not described.

The purpose of this study was 1) to develop protocols for protoplast isolation from embryogenic suspension cultures of avocado, 2) to define optimum conditions for culture and somatic embryos regeneration from the protoplasts.

Materials and Methods

Plant Materials

Plant material used for protoplast isolation consisted of 8–12-day-old avocado embryogenic suspension cultures maintained with a 2-week subculture interval. Details of culture initiation, maintenance, plant growth media and culture conditions are described in Chapter 3.

The embryogenic suspension cultures used in these experiments had been maintained for 1–3 years and their morphologies had changed over time. Initially, the cultures consisted of proembryonic masses that produced secondary somatic embryos repetitively and proembryonic masses. The cultures later consisted primarily of dedifferentiated proembryonic masses ('Esther', 'M25864' and 'Lamb'), mixtures of dedifferentiated, dedifferentiating and highly organized proembryonic masses ('T362', nucellar-derived 'T362', 'Thomas' and nucellar-derived 'Thomas') and highly organized proembryonic masses ('Isham' and nucellar-derived 'Hass').

Protoplast Isolation

Approximately 0.8–1.2 g of 8–14 day-old avocado embryogenic cultures were incubated in a mixture consisting of 2.5 ml of either 0.7 M MS8P$^-$ or 0.7 M MS$^-$8P protoplast culture medium (Table A-2 and A-3) and 1.5 ml enzyme digestion solution (Table A-4) in 60 x 15 mm sterile plastic Petri dishes and sealed with Nescofilm (Grosser & Gmitter, 1990).

The digestion mixtures were incubated in darkness at 25°C on a rotary shaker at 50 rpm overnight (15–18 h). They were then passed through a sterile 45 µm mesh stainless steel screen to remove undigested cell clumps and debris. The filtrates were transferred into sterile 15 ml screw cap centrifuge tubes and precipitated by centrifugation at 100 x g

for 5 min in a clinical centrifuge. After the supernatants were removed, the protoplast pellets were purified by gradient centrifugation using CPW25S and CPW13M (Table A-5b) according to Grosser & Gmitter (1990). The protoplasts at the interphase were collected with Pasteur pipettes and transferred to 15 ml sterile centrifuge tubes and washed once with either 0.7 M MS⁻8P or MS8P⁻ protoplast medium. The protoplasts were then repelletted and resuspended with 0.7 M protoplast medium to a volume of 20x for further studies.

Protoplast yields from several protoplast isolation trials with 'T362', 'Thomas', 'M25864' and 'Esther' were determined using a Fuchs Rosenthal haemocytometer. Protoplast viability was measured using membrane staining fluorescence diacetate (FDA) according to Huang et al. (1986). Approximately 10 µl aliquot of a 10 $mg \cdot ml^{-1}$ stock solution of FDA in acetone was added to 1 ml protoplast suspension. Viable protoplasts were viewed with a fluorescence microscope (Nikon) using 365 nm illumination, and the number of protoplasts was determined by counting with a haemocytometer. Protoplast viability was also determined using the dye exclusion method with Evans blue (Gahan, 1989). Protoplast suspension samples (1–2 ml) were pelleted by centrifugation and resuspended with Evans blue (0.1% w/v) in 0.7 M MS⁻8P protoplast medium for 5 min, and rinsed and resuspended in fresh protoplast medium of the same composition. Protoplast number was determined by counting under a light microscope. Frequency of viable protoplasts consisted of the number of transparent protoplasts divided by the total number of transparent and blue protoplasts.

Effect of Protoplast Density and Medium Osmolarity on Growth of Avocado 'T362' Protoplasts Plated in Agarose Disc Type Medium

A factorial experiment was designed to determine the optimum treatment among four concentrations of medium osmolarity (0.4, 0.5, 0.6 and 0.7 M, each containing 0.15 M sucrose balanced with mannitol) and three protoplast plating densities (4×10^5, 1×10^5

and 0.25 x 10^5 protoplasts ml^{-1} medium). The basal media ware MS8P$^-$ solidified with 20 g l^{-1} agarose type VII (Sigma) that was dissolved in a microwave oven followed by autoclaving for 15 min at 1.1 kg cm^{-2} and 121° C. The media were kept liquid at 45° C until use.

One half ml of protoplast suspension stock at 10 x of a certain treatment density was mixed with 4.5 ml of medium of known osmolarity and then plated on 60 x 15 mm sterile plastic Petri dishes. There were 9 discs in each Petri dish, each consisting of 2 drops of protoplast-agarose medium. When the agarose medium solidified, it was soaked with 3–4 ml liquid medium of the same composition. The Petri dishes were sealed with Nescofilm and maintained in darkness at 25°C.

Plating efficiency as measured according to the equation [Σ microcallus x (Σ microcallus + Σ nondividing protoplasts)$^{-1}$] x 100% was determined from 2 different places on a disc (center and periphery) from 2 discs from 3 Petri dishes (3 replications) of each treatment 3 weeks after culturing.

The microcallus had various two-dimensional shapes: elliptical, rectangular, square, circular, etc. that made it difficult to accurately measure the area. Therefore, the approximate size of the microcallus was determined from the length and width measurement using a Leitz Diavert inverted microscope. Microcalli that were clearly visible in a single focal plane were measured from one disc or as many as 12 microcalli from 3 replications.

The data were analyzed statistically using Proc GLM (SAS Institute, 1992). Plating efficiency data were transformed with arch sine transformation for analysis, and nontransformed data were presented graphically.

Effect of Nitrogen Source, Medium Osmolarity and Protoplast Density on the Growth and Development of Avocado 'T362' Protoplasts in Liquid Medium

A factorial experiment was designed to test the effects of 3 growth parameters of protoplast cultures at 2 levels in liquid growth medium. Nitrogen sources were provided by media MS$^-$8P and MS8P$^+$, in which the total nitrogen content of the former was 61.2 mM, with 18.8 mM from NO_3^- and 42.4 mM from organic NH_4^+(glutamine), while the latter had 60.0 mM nitrogen, with 39.4 mM from NO_3^- and 20.6 mM from inorganic NH_4^+. Medium osmolarity was either 0.6 or 0.4 M (0.15 M sucrose balanced with 0.45 or 0.25 M mannitol, respectively). The protoplast densities were either 0.8×10^5 or 1.6×10^5 protoplasts ml^{-1}. The protoplasts were cultured in 2 ml liquid medium in 60 x 15 mm sterile plastic Petri dishes sealed with Nescofilm and maintained in darkness at 25°C.

Growth of protoplasts was determined by counting the number of microcalli or globular somatic embryos in 2 adjacent squares (4 mm^2) in the center of a Petri dish after the Petri dish had been swirled to obtain an even distribution of microcalli/globular proembryonic masses. The number of microcalli and globular proembryonic masses were counted from three different areas of a Petri dish (left, center and right), after it was swirled to obtained an even distribution of microcallus/globular proembryonic masses, from 3 Petri dishes per treatment. Observations were made 1 month after culture.

The data on the number of microcalli and globular proembryonic masses were presented as percentages and were transformed with arcsine transformation for ANOVA computation (SAS Institute, 1992) and their non-transformed values were presented graphically. The data on the relative number of microcalli/globular somatic embryos were also analyzed for ANOVA and their means and standard errors were presented graphically.

Plating Efficiency of Nucellar-Derived 'T362' Avocado Protoplasts in 0.4 MS-8P and the Effect of Dilution on Somatic Embryo Development.

Plating efficiency

Protoplasts of 'T362' were cultured in 2 ml of 0.4 M MS-8P with a density of $1x10^5$ ml^{-1} in 60 x 15 mm plastic Petri dishes, sealed with Nescofilm, and incubated in darkness at 25°C. Their growth and development were observed after 1, 5, 8 and 14 days. Three cultures were sampled at each observation, and were transferred to 15 ml centrifuge tubes and pelleted by centrifugation at 100 g for 3–5 min. Some of the liquid medium was pipetted out, leaving only 0.2, 0.2, 0.3 and 0.5 ml for observation after 1, 5, 8 and 14 days, respectively. The protoplasts and microcalli were categorized into several groups based on aggregation, necrosis and level of division, and were counted using a Fuchs Rosenthal haemocytometer. Data were presented as percentages (means and standard error) of cells/microcallus within each category (see Results, Table 5-6) and plating efficiency was calculated as the sum of the percentage from categories of cells and clusters of cells that underwent division.

Effect of time and level of dilution on the development of somatic embryos

A factorial experiment was carried out to determine the effect of subculture time and dilution rate of cultured protoplasts on the development of somatic embryos. 'T362' protoplasts were cultured in 2 ml of 0.4 M MS-8P with a density of $1x10^5$ ml^{-1}. At 14, 21 and 28 days after culture, protoplast-derived cultures were subcultured in 2 ml liquid 0.15 M MS-8P with a density of 1/3–1/96 of the original cultures to make dilutions of 3, 6, 12, 24, 48 and 96-fold, respectively.

Protoplast-derived culture morphologies were not the same at the time of subculture. At 14 days after culture, the cultures consisted of mostly globular proembryonic masses ca. 75 μm diameter. At day 21, the globular proembryonic masses were larger (200–300 μm) and in clumps of 0.5–1.0 mm. At day 28, the proembryonic masses were dedifferentiating and were ca. 200–300 μm and in clusters of 1.3–1.5 mm.

After 1 month of culture, 3 replicates (3 Petri dishes) from each dilution treatment and subculture time were randomly sampled and observed. The number of somatic embryos ≥2 mm diameter were counted from each replicate (1 dish) under a dissecting microscope and each culture was weighed.

The data were analyzed as a factorial experiment. Analysis of variances were computed using Proc GLM (SAS Institute, 1992). Means and standard errors were calculated and were plotted in graphs.

Effect of Genotype on Protoplast Yield and Culture Development in Liquid 0.4 M MS-8P Medium

Protoplasts from several genotypes were isolated and purified as described above. They were cultured in 2 ml of 0.4 M MS-8P medium in 60 x 15 mm plastic Petri dishes with a plating density of 0.8–1.6 protoplasts ml^{-1}. The cultures were sealed with Nescofilm and maintained in darkness at 25°C. The morphology of embryogenic cultures at the time of protoplast isolation, their yields and the presence of microcallus after 1 month of culture were recorded.

Somatic Embryo Maturation and Germination on Semisolid Medium

Globular somatic embryos or proembryonic masses (≥1.5 mm diameters) that developed after 1–2 months of culture from 4-week-old 'T362' protoplast cultures on 0.4M MS-8P protoplast medium that had been diluted 20-fold and subcultured in 2 ml M 0.15 MS-8P were used as inocula for observing somatic embryo development on semisolid medium. The inocula, each weighing ca 0.01 g, were transferred to SED medium (Table A-1). There were 9 inocula per Petri dish (150 x 20 mm), which were sealed with Parafilm and maintained in darkness at 25°C

After 1–2 months of culture, opaque somatic embryos (≥0.8 cm diameter) were transferred individually onto SEMG medium (Table A-1) and subcultured at two-month-intervals thereafter (see Chapter 4 for details).

Results

The protoplast yield from avocado embryogenic suspension cultures composed of proembryonic masses (PEM) ranged from 2–30 x 10^6 protoplasts g^{-1} fresh weight, depending on the genotype (Table 5-1). PEM-derived avocado protoplasts were mostly ca 20 μm diameter, although the range was ca. 15–27 μm diameter with a few (< 0.5–1%) being ca. 50 μm diameter. Starch grain content varied with respect to genotype. For example, 'Thomas' and 'M25864' protoplasts had abundant starch grains, while 'Esther' had relatively fewer and 'T362' and nucellar-derived 'Thomas' had virtually no starch grains. Protoplast viability as determined by FDA and Evans blue tests 24 h after isolation and purification was also genotype-dependent. The FDA test consistently produced a value 20% lower than the Evans blue test. Protoplast viability of ca. 80% (FDA test) was observed from 'T362'and nucellar-derived 'Thomas' (Figure 5-1).

Effect of Protoplast Density and Medium Osmolarity on Growth of Avocado 'T362' Protoplasts Plated on Agarose Disc Type Media

Microcalli developed from dividing protoplast-derived cells after 21 days in agarose disc media. Plating efficiency of avocado 'T362' protoplasts 21 days after culture was significantly affected by protoplast plating density, medium osmolarity and their interaction (Table 5-2). Among those factors, plating density appeared to be the most important factor, since its contribution to the variability of the model was 88% as measured by its sum of squares. Figure 5-2 showed that at a plating density of 0.25 x 10^5 protoplast ml^{-1}, irrespective of medium osmolarity, plating efficiencies were significantly lower than with other plating densities. Plating efficiencies at plating densities 1 and 4 x

10^5 were generally high (35–47%) even though their variations were statistically significantly affected by medium osmolarity.

Table 5-1. Protoplast yield per gram fresh weight of avocado embryogenic suspension cultures derived from zygotic embryos of four avocado cultivars.

Cultivars	Yield (Means±Se X 10^6)	Number of Trials
T362	3.50 ± 0.85^1	4
Thomas	30.38 ± 3.73	4
M25864	7.20 ± 1.21	4
Esther	33.00	1

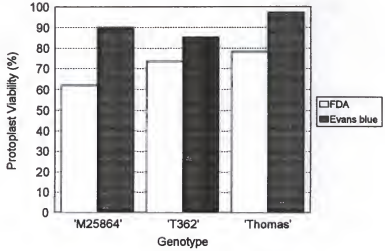

Figure 5-1. Viability of protoplasts isolated from embryogenic cultures derived from zygotic embryos of different avocado genotypes. The viability tests were conducted after storage of protoplasts on ice for 24 h.

Table 5-2. ANOVA of the effect of medium osmolarity and protoplast density on the plating efficiency, length and width of microcalli derived from 'T362' avocado protoplasts cultured in agarose disc type method, three weeks after culture.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Plating efficiency					
Osmolarity (O)	3	435.019	145.006	5.64	0.0051
Density(D)	2	8846.277	4423.139	172.03	0.0001
O*D	5	457.427	91.485	3.56	0.0165
Error	22	565.667	25.712		
Total	32	10041.109			
Length of microcallus					
Osmolarity (O)	3	2154.585	718.195	15.54	0.0001
Density (D)	2	1128.476	564.238	12.21	0.0001
O*D	5	1231.1754	246.235	5.33	0.0002
Error	22	5129.000	46.207		
Total	32	9459.844			
Width of microcallus					
Osmolarity	3	1608.619	536.206	13.42	0.0001
Density	2	1018.1524	509.076	12.74	0.0001
O*D	5	892.773	178.555	4.47	0.0010
Error	22	4435.000	39.955		
Total	32	7871.967			

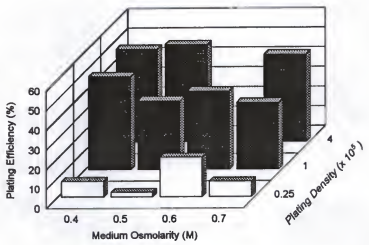

Figure 5-2. Effect of medium osmolarity and protoplast plating density on plating efficiency of 'T362' avocado protoplasts cultured in agarose disc type method after three weeks.

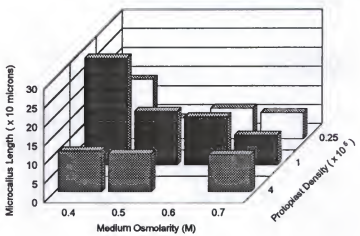

Figure 5-3. Effect of medium osmolarity and protoplast plating density on length of microcalli that developed from 'T362' avocado protoplasts cultured in agarose disc type method after three weeks.

The size of microcalli as represented by their lengths and their widths also was significantly affected by the treatments and their interaction (Table 5-2). At plating densities of 0.25 and 1 x 10^5 protoplasts ml^{-1}, higher medium osmolarity significantly reduced the size of microcalli. At a higher plating density (4 x 10^5 protoplasts ml^{-1}), medium osmolarity has no effect on the size of microcalli. The length and the width of microcalli responded similarly to the treatments; therefore only microcalli length was presented graphically (Figure 5-3). The largest microcalli (280 μm) occurred with a treatment combination of 1 x 10^5 protoplasts ml^{-1} and medium osmolarity of 0.4 M, and this was determined as the best treatment combination since it also yielded a high plating efficiency.

The difference in plating efficiencies and microcallus size as affected by protoplast plating densities when the protoplasts were cultured at medium osmolarity of 0.4 M are depicted in Figure 5-4. A high plating density (4 x 10^5 protoplast ml^{-1}) with a high plating efficiency resulted in a very high number of microcalli per agarose disc (Figure 5-4 A). Optimal plating density (1 x 10^5 protoplasts ml^{-1}) and a high plating efficiency resulted in a lower number but greater size of microcalli (Figure 5-4 C-D) whereas a high plating density resulted in high plating efficiency. A low plating density of 0.25 x 10^5 protoplasts ml^{-1} with a low plating efficiency resulted in few microcalli per disc (Figure 5-4 B).

Small microcalli were also observed in the liquid medium surrounding the agarose medium (Figure 5-3 E), indicating that avocado protoplasts could also be grown in liquid medium.

Replacement of liquid medium in the treatment combination 0.4 M and 1 x 10^5 protoplast ml^{-1} with medium of the same composition but with lower osmolarity (0.15 M) at 3 weeks post-culture resulted in the development of organized proembryonic masses (Figure 5-4 F) from 50% of microcalli ca. 3 weeks later, while no organized growth was observed in medium of unaltered osmolarity. Proembryonic masses also developed from

Figure 5-4. The growth and differentiation of embryogenic culture-derived protoplasts of zygotic-derived 'T362' cultured in agarose medium with medium osmolarity of 0.4 M as affected by plating density. An agarose disc containing microcalli from protoplasts cultured with plating density of 4×10^5 (A), 0.25×10^5 (B) and 1×10^5 protoplasts ml^{-1}. Note the differences in size and density of microcalli in an agarose disc. (D) A higher magnification of a microcallus from C. Note the microcallus showed organization of the cell proliferation. (E) A microcallus that differentiated in liquid medium surrounding the agarose medium. (F) organized proembryonic masses developed from a microcallus after replacing the liquid medium of 0.4 M with 0.12 M liquid medium of the same composition. (G) Organized proembryonic masses from a microcalllus in liquid medium after treatment similar to that of F. (H) Organized proembryonic masses and globular somatic embryos in agarose discs.

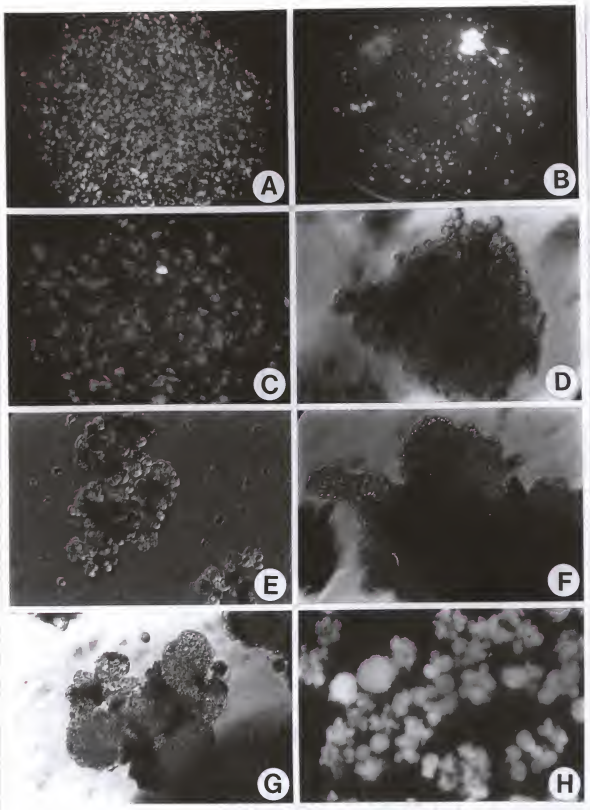
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Figure 5-5 A culture dish containing proembryonic masses and somatic embryos that differentiated from agarose-embedded protoplasts. Somatic embryos and organized proembryonic masses developed both inside the agarose medium and in the liquid medium surrounding the agarose after 3 weeks in medium with 0.4 M medium followed by replacement of the liquid medium with 0.15 M liquid medium of the same composition for 4-5 weeks.

microcalli in liquid medium (Figure 5-4 G). Numerous somatic embryos and organized proembryonic masses developed in agarose discs ca. 7-9 weeks from initial culture, resulting in rapidly growing cultures (Figure 5-5).

Transfer of the 3-week-old agarose discs containing microcalli from the treatment combination 0.4 M and 1 x 10^5 protoplast ml^{-1} into liquid MSP medium resulted in the establishment of embryogenic cultures after ca. 4 months; however, when the microcalli were transferred to semisolid MS medium containing 13% sucrose, embryogenic cultures were observed only after three weeks, while no growth was observed on semisolid MS medium containing 3% sucrose.

Effect of Nitrogen Source, Medium Osmolarity and Protoplast Density on the Growth and Development of Avocado 'T362' Protoplasts in Liquid Medium

The objective of this experiment was to determine the effects of nitrogen source, medium osmolarity and plating density on the growth and development of 'T362' protoplasts in liquid medium. After one month of culture, protoplasts had developed as either unorganized cell masses (microcalli) or organized proembryonic masses. Both morphologies had a diameter of 50–200 µm. Table 5-3 indicates that medium osmolarity, nitrogen source, plating density and the interaction of osmolarity and nitrogen source significantly affected the relative number of microcalli/proembryonic masses that were formed from cultured protoplasts. Even though plating density also had a significant effect on the growth of microcalli, its contribution to the variability of the model as indicated by its sum of squares was only 2.3%, while the other factors, i.e., medium osmolarity and nitrogen source, contributed as much as 39.1% and 39.6%, respectively. Nitrogen source and osmolarity were therefore the critical factors for relative number of microcalli/proembryonic masses formed from protoplasts, while plating density levels tested were not as critical.

Table 5-3. ANOVA of the effect of nitrogen sources, medium osmolarity and protoplast density on number of microcalli and proembryonic masses from avocado 'T362' protoplasts, one month after culture[z].

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Osmolarity (O)	1	834.26	834.26	205.36	0.0001
Nitrogen (N)	1	846.09	846.09	208.27	0.0001
Density (D)	1	49.59	49.59	12.21	0.0131
O*N	1	326.34	326.34	80.33	0.0001
O*D	1	0.51	0.51	0.13	0.0131
N*D	1	4.59	4.59	1.13	0.9023
O*N*D	1	7.59	7.59	1.87	0.9023
Error	16	65.00	4.06		
Corrected Total	23	2133.99			

[z] Number of microcalli and proembryonic masses were determined by counting the number of those two morphologies inside 2 squares of 2 mm^2 observation fields that were located in the center of the Petri dish. The cultures were swirled to provide an even distribution of microcalli and proembryonic masses.

The best treatment combination that resulted in the highest number of microcalli/globular somatic embryos from protoplasts was medium MS⁻8P with medium osmolarity of 0.4 M (Figure 5-6).

The percentages of microcalli that developed from protoplasts were also significantly affected by medium osmolarity, nitrogen source and their interaction. Plating density and its interaction with other factors were not as critical as osmolarity and nitrogen source for microcalli development; however, it was as significant as other factors and their interactions for affecting the percentage of proembryonic masses that developed from protoplasts (Table 5-4). Figure 5-7 shows very clearly that at medium osmolarity of 0.6 M, regardless of the nitrogen source of the medium and plating density, only microcalli developed from protoplasts. At medium osmolarity of 0.4 M, however, proembryonic mass development was dependent upon the source of nitrogen and protoplast density. Irrespective of the plating density, with medium osmolarity of 0.4 M, medium with glutamine (MS⁻8P) allowed the formation of more proembryonic masses than medium

with NH_4NO_3 (MS8P$^-$). With a treatment combination of 0.4 M MS$^-$8P, the lower plating density (0.8×10^5 protoplast ml^{-1}) allowed the development of more proembryonic masses than the higher plating density (1.6×10^5 protoplast ml^{-1}). Since it is easier to adjust the plating density than to alter the nitrogen content in the medium, the combination of medium osmolarity and nitrogen source of 0.4 M MS$^-$8P was adopted as the standard medium for avocado protoplast culture.

Table 5-4. ANOVA of the effect of medium osmolarity, nitrogen source and plating density on the percentages of microcalli and somatic embryo development from avocado 'T362' protoplasts, one month after culture[z].

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Percent microcalli					
Osmolarity (O)	1	1.886	1.886	107.82	0.0001
Nitrogen (N)	1	1.037	1.037	59.58	0.0001
Density (D)	1	0.135	0.135	7.78	0.0131
O*N	1	1.037	1.037	59.58	0.0001
O*D	1	0.135	0.135	7.78	0.0131
N*D	1	0.000	0.000	0.02	0.9023
O*N*D	1	0.000	0.000	0.02	0.9023
Error	16	0.278	0.017		
Corrected Total	23	4.499			
Percent proembryonic masses					
Osmolarity (O)	1	1.876	1.876	107.82	0.0001
Nitrogen (N)	1	1.037	1.037	59.58	0.0001
Density (D)	1	0.135	0.135	7.78	0.0131
O*N	1	1.037	1.037	59.58	0.0001
O*D	1	0.135	0.135	7.78	0.0131
N*D	1	0.000	0.000	0.02	0.9023
O*N*D	1	0.000	0.000	0.02	0.9023
Error	16	0.278	0.017		
Corrected Total	23	4.498			

[z]The data represented the percentage of microcalli and proembryonic masses. The percentage data were transformed with arcsine transformation for the analysis.

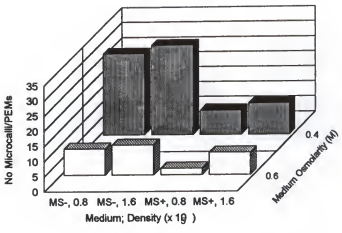


Figure 5-6. Effect of nitrogen source, medium osmolarity and protoplast plating density on the number of microcalli and proembryonic masses that developed from 'T362' avocado protoplasts after one month in culture. MS- = MS⁻8P, MS+ = MS8P⁺, 0.8 indicates 0.8 x 10^5, 1.6 indicates 1.6 x 10^5.

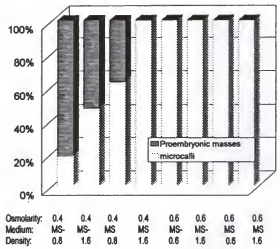


Figure 5-7. Effect of nitrogen source, medium osmolarity and protoplast plating density on the percentage of microcalli and proembryonic masses that developed from 'T362' avocado protoplasts after one month in culture. MS- = MS⁻8P, MS+ = MS8P⁺, 0.8 indicates 0.8 x 10^5, 1.6 indicates 1.6 x 10^5.

Plating Efficiency of Avocado 'T362' Protoplasts in 0.4 MS⁻8P and the Effect of Dilution on Somatic Embryo Development

Plating efficiency and development of proembryos from protoplasts

An objective of this experiment was to observe the growth and development of protoplasts in the standard avocado protoplast liquid medium in order to measure the response of protoplasts at early stages in culture. The results are presented in Table 5-5.

Table 5-5. Percentage of protoplasts that divided on day 1, 5, 8 and 12 after culture[z].

	Day 1	Day5	Day 8	Day 12
	.....(protoplasts ± S.E.) %.....			
Singulated protoplasts				
shriveled/brown	0.0 ± 0.0	19.9 ± 2.0	24.0 ± 2.0	31.5 ± 3.4
healthy/transparent	48.9 ± 7.0	19.2 ±1.6	17.6 ± 1.3	3.4 ± 1.6
1st division (2 cells)	0.0 ± 0.0	1.5 ±0.8	3.7 ± 2.2	0.7 ± 0.7
2nd division (3–4 cells)	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.1	0.4 ± 0.4
3rd division (5–8 cells)	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3	0.4 ± 0.4
proembryos (≥9 cells)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.4 ± 0.4
Cluster of budded or chained protoplasts				
shriveled/brown	0.0 ± 0.0	0.0 ± 0.0	15.1 ± 0.4	26.1 ± 2.7
healthy/transparent	51.2 ± 7.0	56.1 ± 3.6	33.6 ± 2.6	15.1 ± 4.5
1st division (2 cells)	0.0 ± 0.0	3.6 ± 0.5	3.8 ± 1.0	11.2 ± 0.2
2nd division (3–4 cells)	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.3	7.8 ± 1.4
3rd division (5–8 cells)	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	1.7 ± 0.4
proembryos (≥9 cells)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	1.7 ± 1.1
Plating Efficiency	0.0 ± 0.0	5.1 ± 0.6	9.2 ± 1.5	25.0 ± 1.2

[z] Protoplast were cultured in 2 ml liquid medium of 0.4M MS⁻8P with plating density of 1 x 10^5 protoplast ml^{-1}.

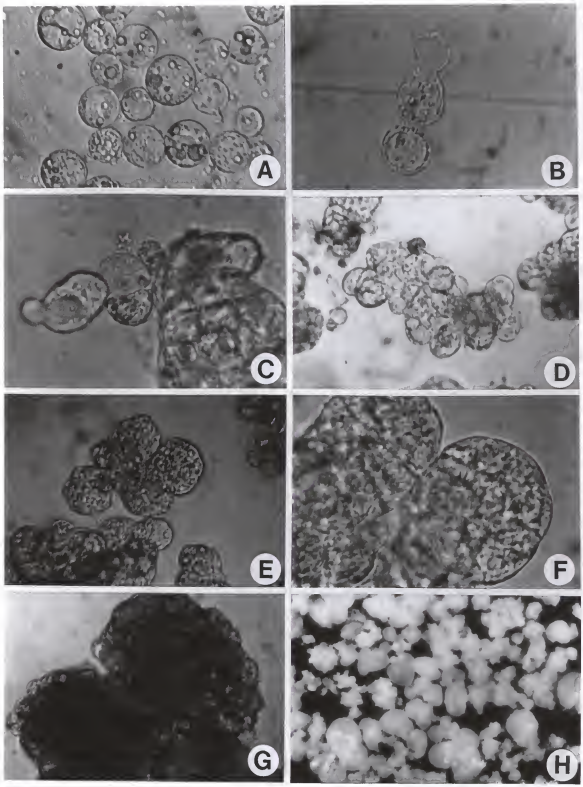
After one day in culture, newly isolated protoplasts (Figure 5-8 A) began to clump together, forming chains or clusters of 3–16 protoplasts (Figure 5-8 B). Some of these clusters or chains disassociated when the cultures were resuspended for observation with a haemocytometer. Protoplast budding was observed in ca. 51% of the samples (Figure 5-8 B and C). Elongation and the beginning of cell division were also observed in some of the protoplasts/cells.

After five days in culture, the protoplasts/cells were grouped into two main categories: singulated protoplasts/cells, and clustered, budded and chained (designated as clustered) protoplasts/cells. Approximately 20% of the singulated protoplasts failed to survive and turned brown, while some (19.2%) remained healthy and transparent; only 1.5% of the protoplasts underwent the first division (Figure 5-8 C). No entirely shriveled and brown clustered protoplasts/cells were observed, and most of them appeared to be alive; however, only ca 3.6% of the protoplasts/cells underwent the first division. The first cell division was observed in one or more cells from the clustered, chained, and budded cells, indicating the single cell origin of the microcalli or proembryonic masses that develop from protoplasts. The percentage of cells/cluster of cells undergoing their first division 5 days after culture was 5.1%.

At day 8, the singulated and the clustered cells also underwent the second and, subsequent divisions (Figure 5-8 D). Clustered cells began to die and turned entirely brown at this time (ca. 15%), and the percentage of singulated protoplasts that failed to survive also increased. After 12 days, the culture morphologies resembled those observed 8 days after culture; however, the plating efficiency was variable. Plating efficiencies at days 5, 8 and 12 after culture were 5, 9 and 25%, respectively (Table 5-5).

Microscopic observation revealed that protoplast-derived cell divisions produce cell clusters that are very tightly organized (Figure 5-8 E and F) demonstrated by the roundness of the cell clusters. Continued observation indicated that these cell clusters

Figure 5-8. Somatic embryogenesis from avocado protoplasts cultured in liquid medium. Embryogenic culture-derived 'T362' protoplasts were cultured in 2 ml liquid medium consisting of 0.4 M MS'8P in 60 x 15 ml Petri dishes after 0–21 days in culture. (A) Freshly isolated protoplasts. (B) Chained protoplast, after 5 days in culture. (C) Budding and a dividing (1st division) protoplast after 5 days. (D) Cluster of cells, some of which underwent 2nd–3rd division forming proembryos. (E) Globular proembryos in clusters, after 14 days in culture. (F) A higher magnification of E. (G) Dedifferentiating proembryos after 21 days of culture. (H) globular somatic embryos or proembryonic masses attained size of 1-3 mm after subculture at medium of lower osmolarity (0.15 M) and low density.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gave rise to globular somatic embryos (Figure 5-8 E & F) which were morphologically different from microcalli (Figure 5-4 D-E). The globular somatic embryos started to dedifferentiate after 21 days of culture (Figure 5-8-G).

Dilution experiment

One month after subculture at diluted culture densities, these cultures were composed of different sizes of globular somatic embryos and microcalli (Figure 5-8 H). The globular somatic embryos ranged 0.15–30 mm diameter and the microcalli were ca 0.5–1.5 mm diameter. Analysis of variance indicated that subculture age, dilution rate and their interaction significantly affected the number of somatic embryos and the fresh weight per Petri dish (Table 5-6).

Table 5-6. ANOVA of the effect of subculture age and dilution rate on culture fresh weight and number of somatic embryos that developed from nucellus-derived 'T362' protoplasts, one month after culture.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Fresh weight					
Age	5	0.045	0.022	13.87	0.0032
Dilution rate	2	0.246	0.049	6.28	0.0001
Age*Dilution rate	10	0.179	0.018	5.04	0.0001
Error	67	0.227	0.004		
Corrected Total	84	0.713			
Number of somatic embryos ≥2 mm					
Age	5	519.369	259.685	111.63	0.0001
Dilution rate	2	123.568	24.714	10.62	0.0001
Age*Dilution rate	10	326.086	32.608	14.02	0.0001
Error	67	155.867	2.326		
Corrected Total	84	1187.812			

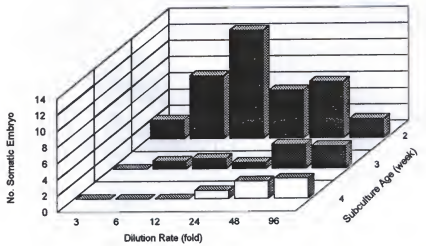

Figure 5-9. Effect of subculture age and dilution rate in medium of low osmolarity (0.15 M MS-8P) on the formation of large (≥ 2 mm diameter) somatic embryos.

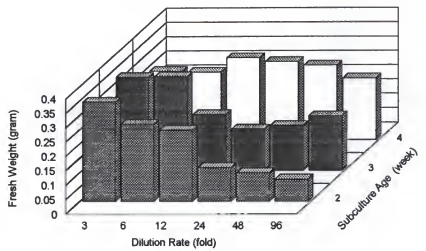

Figure 5-10. Effect of subculture age and dilution rate in medium of low osmolarity (0.15 M MS-8P) on fresh weight accumulation of protoplast-derived proembryos after one month of culture.

Subculture age was very critical for development of somatic embryos ≥2 mm diameter. When protoplast cultures were subcultured with diluted density at day 15, dilution rates of 6–48-fold resulted in the formation of 6–12 globular somatic embryos per plate. When the protoplast-derived cultures were subcultured at diluted density at days 21 and 28, high dilution rates (49–96-fold) were required for the development of somatic embryos with diameter ≥2 mm, yet at low frequency (maximum 5 somatic embryos per plate) (Figure 5-9); however, abundant somatic embryos ≤2 mm were observed in addition to disorganized proembryonic masses.

Figure 5-11 indicated that the fresh weight of cultures decreased with respect to dilution rate, when cultures were subcultured at day 15. A similar trend was also observed for subculture at day 21, except that fresh weight of cultures was higher than that of cultures subcultured at day 14 with dilution rates of 6-fold and higher. With subculture at day 28 the dilution rate had virtually no effect on culture fresh weight. At high dilution rates (12–96 fold), the fresh weight of cultures that were subcultured at day 28 was much higher than that of cultures subcultured at earlier ages. Nevertheless, the fresh weights of cultures that were subcultured at day 28 were as much as the highest fresh weight that could be obtained by other cultures that were subcultured earlier and at lower dilution rate, i.e., ca. 2.5–3.3 gram per Petri dish.

Effect of Proembryonic Mass Morphology on Protoplast Yields and Responses in Liquid 0.4 M MS⁻8P Medium

Table 5-7 has summarized the variation in growth and development responses of avocado protoplasts isolated from proembryonic masses of different morphologies and genotypes in standard liquid avocado protoplast medium (0.4 M MS⁻8P) with standard plating density (1×10^5 protoplasts ml^{-1}). When the proembryonic masses used for protoplast isolation were from newly established suspension cultures at which time the proembryonic mass was largely nodular ('Booth 8', 'Isham', 'Hass'), low protoplast yields

were obtained. This type of protoplast did not divide and was necrotic after 3–4 weeks. High protoplast yields were recovered from suspension cultures that consisted of a mixture of nodular proembryonic masses and disorganized proembryonic masses

Table 5-7. Relationship among genotypes, their embryogenic characteristics, protoplast yield and response after one month in liquid medium 0.4 M MS-8P, at plating density of 0.8–1.2 x 10^5 protoplast ml^{-1}.

Cultivar/ Genotype of Mother Tree	Type of Embryogenic Culture[a]	Morphology of PEMs at Time of Protoplast Isolation[b]	Protoplast Yield[c]	Response: microcalli or PEMs or Both
'Booth 8'	late dedifferentiating	nodular	low	none
'Esther'	early dedifferentiating	disorganized	high	microcalli
'M25864'	early dedifferentiating	disorganized	high	microcalli
'Isham'	no dedifferentiating	nodular	low	none
'Thomas' (zygotic)	intermediate	mixture	high	microcalli/PEMs
'Thomas' (zygotic)	intermediate	disorganized	high	microcalli/no growth
'Thomas' (nucellar)	late dedifferentiating	mixture	high	microcalli/PEMs
'Thomas' (nucellar)	late dedifferentiating	disorganized	high	no growth
'Hass'	late dedifferentiating	nodular	low	no growth
'Hass'	late dedifferentiating	mixture	high	microcalli
'Lamb'	late dedifferentiating	nodular	low	N.A[d].
'Lamb'	late dedifferentiating	disorganized	high	microcalli
'T362'	late dedifferentiating	mixture	high	microcalli/PEMs

[a] Type of embryogenic culture was determined from newly initiated embryogenic culture in liquid medium or from newly initiated embryogenic culture on semi solid medium.

[b] Morphologies of cultures at time of protoplast isolation may have changed from their initial morphologies.

[c] High yield if the volume of pelleted protoplast after gradient centrifugation was ≥0.01 ml, low yield if the volume of pelleted protoplast after gradient centrifugation was 0.01–0.03 ml. Approximately 1 g fresh weight of proembryonic masses was used for protoplast isolation,

[d] Protoplast culture was not attempted.

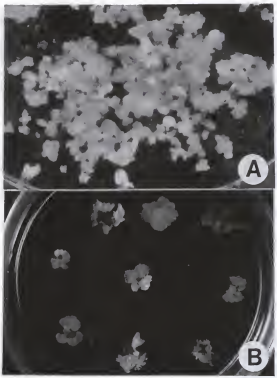

Figure 5-11. Somatic embryo development from protoplasts derived cultures as affected by culture method. (A) Numerous but small somatic embryos developed when the entire content of protoplast culture disc was poured onto a dish with SED medium. (B) Large and maturing somatic embryos developed when organized proembryonic masses were used as inoculum in a similar manner to that described for somatic embryo development (Chapter 4). The scale in A is twice of B.

('T362', mixture of disorganized and nodular PEMs of 'Thomas' from zygotic or nucellar origin). These protoplasts developed into microcalli and globular somatic embryos after one month of culture. Consistently high yields were obtained when completely dedifferentiated proembryonic masses were used for protoplast isolation. These protoplasts, however, only developed as microcalli and had lost their morphogenic potential (disorganized proembryonic masses from 'Lamb'), sometimes budding and forming chains of dividing cells without further division (disorganized 'Thomas' proembryonic masses).

Somatic Embryo Development, Maturation and Germination

In attempting to obtain good quality somatic embryos that would develop normally to germination, the method that was developed for citrus (Grosser & Gmitter, 1990) was tried. Accordingly, the entire culture (from 1 culture dish) that consisted of enlarged nodular proembryonic masses (1–2 mm) and smaller proembryonic masses was poured onto semisolid SED medium in a 110 x 20 mm Petri dish. After 1 month of culture, a mixture of small (≤0.5 mm diameter), opaque and hyperhydric somatic embryos developed and covered almost the entire surface of the SED medium (Figure 5-11 A). These somatic embryos did not enlarge further and did not germinate after transfer to germination medium. Alternatively, only the large (1–2 mm) protoplast-derived nodular proembryonic masses and dedifferentiating proembryonic masses were used as inocula on semisolid medium. After 1–2 months of culture, proembryonic masses as well as somatic embryos of different sizes, different stages of development and of varying hyperhydricity developed from the inoculum (Figure 5-11 B).

Only opaque somatic embryos ≥0.8 cm diameter were transferred individually to somatic embryo germination medium. The somatic embryos enlarged to ca 1.0–1.5 cm diameter. After transfer onto germination medium, ca. 60% of the cultures produced

secondary somatic embryos and/or disorganized proembryonic masses, whereas the remainder continued to enlarge. After 4 successive transfers, no shoot development was observed from ca. 80 somatic embryos.

Discussion

Avocado protoplasts could be isolated and purified with consistently high yields from embyogenic suspension cultures consisting of dedifferentiating proembyonic masses using a protocol that has been used routinely for citrus (Grosser & Gmitter, 1990), and for grape and mango (J. W. Grosser, personal communication). Avocado protoplast yields consisting of 3–30 x 10^6 protoplasts g^{-1} fresh weight were as high as the yields of other species, e.g., citrus (Grosser and Gmitter, 1990; Vardi et al., 1987), pear (Ochatt & Power, 1988), sour cherry (Ochatt, 1990), peach (Matsuta et al., 1986; Mills & Hammerschlag, 1994) and *Coffea arabica* (Grèzes et al., 1994).

Protoplasts isolated from embryogenic 'T362' avocado cultures developed directly into proembryos and subsequently into nodular proembryonic masses and later heart stage and more mature stage somatic embryos when the physical and chemical cultural conditions were optimized. This direct pathway of somatic embryo regeneration from protoplasts derived from embryogenic cultures has been reported for somatic hybrids of citrus (Grosser & Gmitter, 1990), including *Citrus sinensis* (Kobayashi et al., 1985; Niedz, 1993), *Citrus mitis* (Sim et al., 1988), *Citrus madurensis* (Ling et al., 1989), *Citrus unshiu* (Ling et al., 1990) and grapevine (Reustle et al., 1995). Indirect somatic embryo development from embryogenic culture-derived protoplasts has been reported for citrus and citrus relatives (Kunitake et al., 1991; Jumin & Nito, 1995, 1996a, b), and involves regeneration after protoplast-derived microcallus has been transferred to medium containing plant growth regulators. No plant growth regulators are required for direct somatic embryogenesis from avocado protoplast cultures derived from embryogenic

cultures. This indirect pathway of embryogenesis reported for citrus is probably related to the morphogenic potential of the embryogenic cultures from which the protoplasts were isolated. In those studies, the protoplasts were isolated from embryogenic cultures established from seedling stem instead of nucellus or ovular tissues, and it was not clear if those embryogenic cultures consisted of organized or disorganized proembryonic masses. Somatic embryo development was not prolific from these cultures (Jumin & Nito, 1995; 1996a; b). Differentiation via organogenesis has also been reported from protoplasts, and is typical of woody fruit species regenerated from leaf mesophyll protoplasts, e.g., pear, *Pyrus* spp., (Ochatt, 1993b), stone fruits (*Prunus* spp.) (Ochatt, 1993c) and apple (*Malus* X *domestica*) (Patat-Ochatt, 1994). The regeneration pathway of a protoplast seems to be determined by the competence of the source tissue, implying the importance of choice of source tissue.

The early stages of development of proembryos, proembryonic masses and somatic embryos from avocado embryogenic protoplasts was affected by cultural parameters that included physical and chemical factors and by genetic and epigenetic characteristics of the protoplast sources. Plating density and medium osmolarity have been reported to be two of the most critical physical parameters for citrus protoplast culture and significantly affect plating efficiency and somatic embryo development (Kobayashi et al., 1985). While optimal plating density of avocado protoplasts at ca 10^5 ml^{-1} is similar to that reported for other species, i.e., citrus (Vardi et al., 1975; Vardi & Galun, 1988; Grosser & Gmitter, 1990), the optimum medium osmolarity of 0.4 M is low compared to the standard 0.6–0.7 M used for other perennial species, i.e., citrus (Grosser & Gmitter, 1990; Hidaka & Kajiura, 1988; Niedz, 1993; Sim et al., 1988; Ling et al., 1990) and grape (Reustle et al., 1995). When protoplasts were cultured at high medium osmolarity (0.6–0.7 M), the osmolarity needed to be reduced progressively, while no weaning was required for avocado and citrus (Kobayashi et al., 1985) cultured at 0.4 M. The beneficial effect of

low medium osmolarity for efficient somatic embryo development has been reported in citrus by Kobayashi et al. (1985).

Omission of NH_4NO_3 from the basal medium has been reported to be beneficial for microcallus development from mesophyll protoplasts of *Populus* (Russell & McCown, 1986, 1988) and of *Pyrus* sp. (Ochatt, 1992; 1993b; Ochatt & Caso, 1986). Grosser (1994) suggested that high NH_4NO_3 concentration in the medium (as in MS) could be toxic to protoplasts; however, glutamine at 42.4 μM has been routinely used as one of the nitrogen sources in citrus protoplast culture medium (Grosser & Gmitter, 1990). Whether the increased frequency of somatic embryo formation from 'T362' avocado protoplasts following replacement of NH_4NO_3 by glutamine is related to the ratio of reduced nitrogen in the medium or to the presence of the organic form of ammonia is not clear. Experiments concerning the effects of the ratio and concentration of inorganic, reduced and oxidized nitrogen indicated that medium with a low ratio or even without reduced nitrogen were beneficial for somatic embryo development from dedifferentiated proembryonic masses (see Chapter 3). In contrast, Higashi et al. (1996) reported that embryogenic carrot cell clusters required reduced nitrogen for somatic embryo development, and organic nitrogen, i.e., glutamine, strongly affected the development of mature somatic embryos.

Development of globular somatic embryos or proembryonic masses of 1–2 mm diameter required subculture of protoplast-derived cultures into lower densities and into medium with low osmolarity (0.15 M). Reduction of density of cells derived from citrus protoplasts resulted in somatic embryo development and caused Kobayashi et al. (1985) to suggest that embryogenic potential might be repressed when cells are cultured at high cell density. Grosser and Gmitter (1990) suggested that cultures with high protoplast plating efficiency should be diluted; otherwise no somatic embryos could be regenerated.

Proembryos and somatic embryos that developed from avocado protoplasts were unable to develop to maturity and germinate after transfer to semisolid medium, unlike

citrus (Grosser & Gmitter, 1990; Kobayashi et al., 1985; Hidaka & Kajiura, 1988; Sim et al., 1988; Niedtz, 1993). As a result, the proembryonic masses or somatic embryos that developed from avocado protoplasts were used as inocula to obtain good quality white, opaque cotyledonary somatic embryos of ≥ 0.8 cm diameter which enlarged to 1.0–15 cm diameter on medium with low osmolarity. This approach has been used to develop good quality somatic embryos from embryogenic suspension cultures of avocado (Chapter 4). The low frequency germination of protoplast-derived mature somatic embryos is probably related to the low plant regeneration from avocado somatic embryos (see Chapter 4; Pliego-Alfaro & Murashige; 1988, Mooney & Van Staden, 1987).

In conclusion, a protocol for simple and efficient isolation, culture and regeneration of mature somatic embryos from protoplasts isolated from avocado embryogenic suspension cultures has been developed for the first time. This protocol could open opportunities for avocado improvement by somatic cell genetics. For example somatic hybridization between avocado and root rot resistant small seeded *Persea* species could be attempted and direct gene transfer protocols could be developed.

CHAPTER 6
AVOCADO SHOOT CULTURE AND PLANTLET DEVELOPMENT AND NET PHOTOSYNTHESIS IN A NON-ELEVATED AND ELEVATED CO_2 ENVIRONMENT

Introduction

In vitro propagation of avocados (*Persea americana* Mill.) from seedling shoot tip and nodal explants has been described with limited success, including some shoot elongation of existing buds, limited shoot proliferation and formation of scaly leaves (Schroeder, 1979, 1980; Young, 1983; Vega-Solarzano, 1989; González-Rosas & Salazar-Garcia, 1984; Harty, 1985; Barringer, 1996). Shoot elongation and formation of expanding leaves were also reported from nodal stem segments of 1-year-old *P. schiedeana* Nees (González-Rosas et al., 1985). Schall (1987) and Cooper (1987) described culture of avocado seedling shoot tips, and subsequently rooted and acclimatized plantlets. Attempts by Pliego-Alfaro & Murashige (1988) to micropropagate adult trees from shoot tips using *in vitro* serial grafting were unsuccessful. Limited shoot multiplication that declined with each subculture and low rooting frequency were reported when shoots were derived from heavily pruned grafted scions derived from mature phase avocado (Pliego-Alfaro et al., 1987). A protocol for rooting of juvenile shoots *in vitro* has been developed (Pliego-Alfaro, 1988).

In addition to their low multiplication rate, survival of shoots *in vitro* was reported to be ca 80% when Murashige & Skoog (1962) (MS) formulation was used as the basal medium (Schall, 1988). On MS medium, avocado shoots developed scaly leaves, leaf tip burn and shoot die back, depending on the genotype. These symptoms were similar to ammonia toxicity symptoms that have been described by Lovatt (1988). On an SM

medium supplemented with activated charcoal, necrosis of the stems in contact with medium has also been observed (Witjaksono, unpublished data).

Despite success in propagating juvenile phase avocado by shoot tip culture, detailed studies regarding acclimatization of *in vitro*-grown plantlets have not been reported (Schall, 1987; Cooper, 1987). Exposing *in vitro*-grown plantlets and seedlings to higher irradiance, low nutrient concentration, and elevated atmospheric CO_2 concentration have been demonstrated to increase net photosynthesis and biomass production (Kozai, 1991) with concommitant increased acclimatization rate of plantlets (Kozai, 1991; Laforge et al., 1991). The photosynthesis of avocado shoots or plantlets *in vitro* has not been reported.

The same protocols described for shoot multiplication of juvenile phase avocado are ineffective for mature phase avocado selections, especially for Phytophthora root rot-tolerant rootstocks; however these protocols could be useful for multiplying breeding lines and germplasm, e.g., multiplication of shoots from immature embryos isolated from abscised fruitlets that resulted from controlled avocado crosses (Skene & Barlass, 1983) and multiplication of shoots from somatic hybrids (Grosser & Gmitter, 1990; Ochatt, 1990). Multiplication of shoots that developed from germinating avocado somatic embryos would be very critical, especially when the germinating somatic embryos are derived from somatic hybridization, genetic transformation or from nucellar explants, since the plant conversion frequency is very low (Pliego-Alfaro & Murashige, 1988; Mooney & Van Staden, 1987; see Chapter 4).

The purpose of the studies described below was to develop a shoot multiplication protocol that would permit high multiplication and survival rates of *in vitro*-grown plantlets and to compare the photosynthetic rate of *in vitro*-grown shoots and plantlets grown in different atmospheric CO_2 concentrations.

Materials and Methods

General Procedures

The pH of all media was adjusted to 5.7–5.8 with either 0.1–1.0 N KOH or HCl, and 8 g l^{-1} TC agar (Carolina Biological Supply Co., 2700 York Rd, Burlington NC 27215-3398) was added thereafter unless specified otherwise. The plant growth medium was melted by autoclaving at 121°C at 1.1 kg cm^{-2} for 5 min, and dispensed in 25 ml aliquots into 150 x 25 mm glass test tubes. The test tubes were capped with polypropylene closures and autoclaved at 121°C at 1.1 kg cm^{-2} for 15 min and cooled as slants unless stated otherwise.

After explant inoculation, the tubes were capped with autoclaved 12 x 12 cm Suncaps® [clear plastic film with a 10 mm gas permeable filter (Sigma Chemical Co., St. Louis, MO)] and secured with rubber bands. The cultures were maintained in an incubator at 25 ± 2°C with an irradiance of 100–120 μmol m^{-2} s^{-1} and a 16 h photoperiod provided by Gro-Lux lamps, regular spectrum (Osram Sylvania Inc., Danvers, MA 01923).

Plant Materials

Shoots derived from juvenile phase proliferating shoot cultures of 'Guaram 13' avocado were provided by Dr. Fernando Pliego-Alfaro, Departamento de Biologia Vegetal, Universidad de Malaga, Malaga, Spain. The shoot cultures were maintained on plant growth medium consisting of (in mg l^{-1}): NH_4NO_3, 719.4; KNO_3, 1816.4; $NaNO_3$, 85; $Ca(NO_3)_2$ 4 H_2O, 944.0; $MgSO_4$ 7 H_2O, 245.3; KH_2PO_4, 136.0; KCl, 372; myo-inositol, 100; MS micronutrients and vitamins; and 4.44 μM BA and sucrose, 30.000. The medium was solidified with 6 g l^{-1} agar (Sigma Chemical Co., St. Louis, MO). The shoot cultures were thereafter maintained in 8-week subculture cycles on a plant growth medium

containing MS major salts devoid of NH_4NO_3, MS minor salts supplemented with modified LS organic addenda and 4.44 μM BA, thereafter referred as Shoot Multiplication (SM) medium (Table A-1).

Effect of KNO_3 on Avocado Shoot Proliferation

Five levels of KNO_3 as the sole inorganic nitrogen source in the medium were tested (20, 30, 40, 50 and 60 mM). Otherwise, the composition of the medium was the same as SM medium. There were 10 tubes (replicates) per treatment. The explants were 'Guaram 13' nodal stem segments 1–1.5 cm in length with 1–4 axillary buds that had been maintained on SM medium for 8 weeks. After 8 weeks, shoot number, shoot length and number of leaves were recorded for plants for data analysis.

Effects of Total Nitrogen Concentration, NO_3^-:NH_4^+ Ratio and Atmospheric CO_2 Concentration on Growth of Shoot Cultures

The total nitrogen concentration and ratio of NO_3^-:NH_4^+ were as follows: 20 mM of NO_3^-:NH_4^+ with a ratio of 1:0, 20 mM of NO_3^-:NH_4^+ with a ratio of 3:1, 40 mM of NO_3^-:NH_4^+ with a ratio of 3:1 and 60 mM of NO_3^-:NH_4^+ with a ratio of 3:1. The nitrogen was supplied as KNO_3 and NH_4NO_3. The remaining composition of the plant growth medium was similar to SM medium. Cultures were grown either in an incubator with ambient atmospheric CO_2 (350 μmol mol^{-1}) or in a plexyglass box with an elevated CO_2 concentration. Irradiance in the incubator and in the box were 120–150 μmol m^{-2} s^{-1} and 150–170 μmol m^{-2} s^{-1}, respectively, provided by Gro-Lux lamps, regular spectrum (Osram Sylvania Inc., Danvers, MA 01923) with a 16 h photoperiod. The box was constructed of 0.55 cm-thick plexyglass with an outer dimension of 107.2 (length) x 50.6 (width) x 21.7 (height) cm. The CO_2 was supplied to the box via tygon tubing from a tank of 2% (v:v) CO_2 in nitrogen. The gas passed through distilled water before entering the box with a flow rate of 11.66–12.56 cm^3 min^{-1}.

The explants for the experiment consisted of 'Guaram 13' nodal stem segments of 1–1.5 cm with 1–4 axillary buds from 2-month-old shoot cultures maintained on SM medium. There were 10 tubes (replicates) per treatment.

After 8 weeks, the number of shoots (<1 cm and ≥1 cm length), length of shoots ≥1 cm, number of small leaves (2–4 mm width) and the number of expanded leaves (>4 mm width) were determined for shoot cultures maintained in the incubator. After 10 weeks of culture, growth variable data similar to those determined at 8 weeks were collected from all cultures in both growth environments. Net photosynthesis and dry matter content were determined for five randomly sampled cultures from each treatment in each atmospheric CO_2 environment (see Photosynthesis Measurements for detail).

Effect of Ambient CO_2 on Plantlet Development

Rooting of shoots was done using a two step method, consisting of induction and development (Pliego-Alfaro, 1988). Induction involved the use of explant shoots (1.5–2 cm long with 1–3 leaf primordia and non-expanded leaves) that were excised from proliferating 'Guaram 13' shoot cultures maintained on SM medium for 2 months. Individual shoots (10–15) were cultured for 3 days in Magenta™ GA7 vessels (Magenta Corporation, Chicago, IL) containing 50–60 ml Root Induction (RI) medium (Table A-1) and capped with sterile transparent Suncaps® (Sigma Chemical Co., St. Louis, MO). The cultures were maintained in an incubator with ambient CO_2 concentration.

After 3 days on RI medium, shoots were individually transferred into 150 x 25 mm test tubes containing ca. 25 ml semi-solid Root Development (RD) medium (Table A-1). The medium was cooled with the tubes remaining upright. There were 60 shoots; 30 shoots were maintained in a growth chamber at ambient atmospheric CO_2 and 30 shoots were maintained in the box with elevated atmospheric CO_2. After 9 weeks, net

photosynthesis was determined for 10 randomly selected cultures from each growth environment, and growth data were collected.

Photosynthesis Measurements

Net photosynthesis was measured for:

1) Proliferating shoot cultures, their shoot cuttings with subtended callus and plantlets and plantlet-derived shoot cuttings. Preliminary experiments indicated that proliferating shoot cultures on which basal callus developed had a negative net photosynthesis, indicating net respiration. Likewise, shoots from which basal callus had been removed showed positive net photosynthesis. To confirm these observations that net negative photosynthesis value of the intact proliferating shoot cultures might have been due to respiring callus masses, net photosynthesis was determined for proliferating shoot cultures (on medium with NO_3^-:NH^+ ratio of 3:1 at 40 mM) maintained in ambient and elevated CO_2 concentrations (4 replicates each). Subsequently, all shoots in proliferating shoot cultures were removed from the basal callus which was subcultured separately. The dissected shoots from a proliferating culture were subcultured for net photosynthesis measurement in a single tube containing fresh medium of the same composition. Net photosynthesis was also determined for the subtended callus that had been removed. Stem, leaf and callus dry weights (24–48 h, 60° C) and leaf area (measured with a Portable Area Meter, Model L1-300, Lambda Instruments Corporation, Lincoln, NE) were recorded.

Dissection of shoots from proliferating cultures may cause wound respiration. To determine if dissection had a significant effect on respiration (and therefore net photosynthesis) of the shoot cuttings, an experiment was conducted using plantlets that developed under elevated CO_2 concentration. Net photosynthesis was measured for four intact plantlets. Subsequently, the plantlets were decapitated 1–2 mm above the root

zone. The resulting shoot cuttings were subcultured individually on RD medium for net photosynthesis measurement. Leaf area, stem and leaf dry weight were recorded.

2) Shoots derived from proliferating shoot cultures from nitrogen concentration, NO_3^-:NH_4^+ ratio and atmospheric CO_2 concentration experiments. Ten weeks after the nodal stem segments were cultured, shoots had proliferated and basal callus had developed where proliferating shoots were in contact with the plant growth medium. Five cultures were randomly sampled from the NO_3^-:NH_4^+ ratio and atmospheric CO_2 concentration experiments. Two to four shoots (2–5 cm length) were removed from each proliferating shoot culture without damaging the leaves and were subcultured onto fresh medium with 40 mM nitrogen with a NO_3^-:NH_4^+ ratio of 3:1 for photosynthesis measurement. Leaf area and fresh and dry weights of the stems and leaves were recorded from the subcultured shoots. Dry matter content (%) of the shoots was calculated using the formula: 100% - [(fresh weight - dry weight): dry weight] x 100%.

3) Plantlets that developed in ambient and elevated CO_2 environment. Ten replicates (test tubes) were randomly sampled from rooted shoot cultures that developed in ambient and elevated CO_2 environments. Net photosynthesis was determined from each tube. Shoot length, number of leaves, leaf area, number of roots and root length and shoot, leaf, root and callus dry weights were recorded.

Net photosynthesis was measured with an ADC, LCA-3 portable gas exchange system under shade with irradiance ca 295–486 μmol m^{-2} s^{-1} at 30°C. The culture tubes were each fitted with a two-holed rubber stopper into which copper tubing had been inserted. The copper tubings served as inlet and outlet ports. The stoppers were sealed to the glass tubes with Parafilm. Air (containing 350 CO_2 μl l^{-1}) was pumped through rubber tubing into the inlet port at a flow rate of 400 ml min^{-1}. Air pressure inside the culture tubes was presumably sufficient to allow mixing of air. Air flowed from the outlet port through rubber tubing to the infrared gas analyzer and the CO_2 depletion rate in the tube was determined. Net photosynthesis was expressed in terms of leaf area or dry weight.

Statistical Analyses

All statistical tests were performed using SAS software for IBM-compatible personal computers, version 6.08 for Windows (SAS Institute, 1992) or SigmaPlot™ (Jandel Scientific, San Rafael, CA). The following statistical analyses were performed:

Effect of KNO_3 concentration on shoot growth in the incubator. The effects of KNO_3 concentration on shoot growth responses were determined by regression analysis.

Effect of NO_3^-:NH_4^+ ratio on shoot growth responses in the incubator after 8 weeks. The effect of two different NO_3^-:NH_4^+ ratios (1:0 and 3:1) on shoot growth responses was compared using standard t-tests.

Effect of N concentration on shoot growth responses in the incubator after 8 weeks. The growth responses of shoots to different N concentrations (NO_3^-:NH_4^+ ratio was 3:1) were determined by regression analysis.

Effects of atmospheric CO_2 and NO_3^-:NH_4^+ ratio on shoot growth responses and photosynthesis after 10 weeks. Interactions between atmospheric CO_2 concentrations and nitrogen ratios (at 20 mM total N in the medium) on shoot growth responses and net photosynthesis were determined by ANOVA. If there were no interactions, data were pooled across CO_2 concentrations to test nitrogen ratio effects and data were pooled across nitrogen ratios to test CO_2 effects. A standard t-test was used to compare growth and photosynthetic responses between a medium containing 20 mM with a NO_3^-:NH_4^+ ratio of 1:0 and a medium providing the same total N concentration with a NO_3^-:NH_4^+ ratio of 3:1.

Growth and photosynthetic responses of shoots in an ambient CO_2 environment were compared to those of shoots grown in an elevated CO_2 concentration with a standard t-test.

Effects of atmospheric CO_2 and N concentration on shoot growth responses and photosynthesis after 10 weeks. Interactions between atmospheric CO_2 concentration and

nitrogen concentration (20, 40 or 60 mM total nitrogen with a NO_3^-:NH_4^+ ratio of 3:1) on shoot growth and net photosynthesis were determined by ANOVA. If there were no interactions, data were pooled across CO_2 concentrations to test nitrogen effects and data were pooled across nitrogen concentrations to test CO_2 effects.

The effects of total nitrogen concentration on shoot growth and net photosynthesis were determined by linear regression analysis. Growth and photosynthetic responses of shoots in an ambient CO_2 environment were compared to those of shoots grown under elevated CO_2 conditions with a standard t-test.

Effect of atmospheric CO_2 on plantlet development after 9 weeks. The effect of atmospheric CO_2 on the growth and development of plantlets was determined by standard t-test.

Results

General Morphology of the Cultures

In addition to single or multiple shoot elongation from the preexisting axillary buds in the nodal explants, soft brown and or compact green calli developed at the cut end of each nodal stem segment propagule that was in contact with medium. After 8–10 weeks, callus covered the entire explant and covered the surface of the medium in some cultures.

Effect of KNO_3 on Growth Responses of Shoot Cultures

The total number of shoots per culture was not significantly affected by KNO_3 concentration in the medium ($P > 0.05$); however, the proportion of healthy or necrotic shoots was significantly different with different KNO_3 concentrations. The number of healthy shoots decreased curvelinearly with increasing KNO_3 concentration (Figure 6-1-A), whereas the number of necrotic shoots increased curvelinearly with increasing KNO_3

concentration (Figure 6-1-B). At 20 mM KNO_3, no necrotic shoots were observed. The length of healthy shoots decreased curvelinearly as KNO_3 concentration decreased (Figure 6-1-C), while the length of necrotic shoots increased with increasing KNO_3 concentration (Figure 6-1-D). The total length of shoots was not significantly affected by KNO_3 concentration ($P > 0.05$).

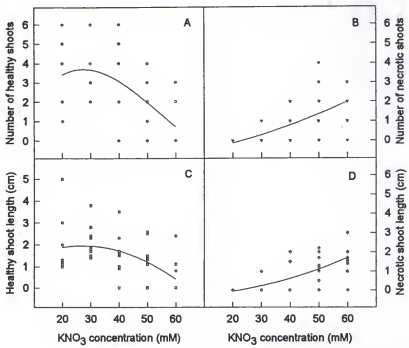

Figure 6-1. *In vitro* shoot growth of 'Guaram 13' avocado in response to varying concentrations of KNO_3 (N) as the sole inorganic nitrogen source in the medium. The growth variables and their regression lines are: A) number of healthy shoots, $Y = -1.04 + 0.39\ N - 0.0095\ N^2 + 0.000058\ N^3$, $r^2 = 0.35$; B) number of necrotic shoots, $Y = -0.76 + 0.02\ N - 0.00036\ N^2$, $r^2 = 0.47$; C) healthy shoot length, $Y = -1.49 + 0.29\ N - 0.0071\ N^2 + 0.000048\ N^3$, $r^2 = 0.28$; D) necrotic shoot length, $Y = -0.3 + 0.0014\ N - 0.00054\ N^2$, $r^2 = 0.50$.

The number of scaly leaves, number of small leaves, number of expanded leaves, number of abscised leaves and total number of leaves decreased curvelinearly with increasing KNO_3 concentration (Figure 6-2-A–E). For all growth variables, the coefficient of correlations (r^2) of the regression lines were low, ranging from 0.28 to 0.56.

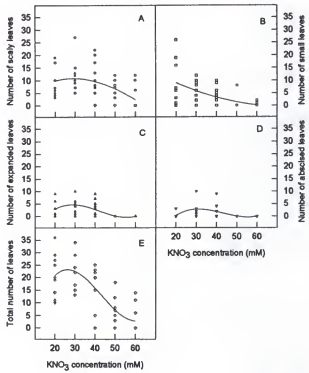

Figure 6-2. *In vitro* leaf growth of 'Guaram 13' avocado shoots in response to varying concentrations of KNO_3 (N) as the sole inorganic nitrogen source in the medium. The growth variables and their regression lines are: A) number of scaly leaves, $Y = -24.46 + 2.89\ N - 0.07\ N^2 + 0.00051\ N^3$, $r^2 = 0.27$; B) number of small leaves, $Y = -17.84 + 0.5\ N + 0.0035\ N^2$, $r^2 = 0.35$; C) number of expanding leaves, $Y = -23.76 + 2.39\ N - 0.06\ N^2 + 0.0005\ N^3$, $r^2 = 0.31$; D) number of abscised leaves, $Y = -25 + 2.19\ N - 0.05\ N^2 + 0.00043\ N^3$, $r^2 = 0.28$; E) total number of leaves, $Y = -32.5 + 4.96\ N - 0.14\ N^2 + 0.0011$, $r^2 = 0.56$.

Effect of NO_3^-:NH_4^+ Ratio on Shoot Growth Response of Cultures in the Incubator after 8 Weeks

Eight weeks after culture, a 3:1 NO_3^-:NH_4^+ ratio resulted in more shoots ≥1 cm ($P < 0.03$), and greater total shoot length ($P < 0.02$) than did a NO_3^-:NH_4^+ ratio of 1:0. The number of shoots >1 cm long and the average shoot length were not significantly different between the two N ratio treatments. Neither treatment significantly affected the number of expanded leaves; however, when the NO_3^-:NH_4^+ ratio was 3:1, significantly more small leaves ($P < 0.01$) and a greater total number of leaves ($P < 0.07$) were observed than for the other treatment (Table 6-1).

Table 6-1. The effect of NO_3^-:NH_4^+ ratio at 20 mM on the growth of 'Guaram 13' avocado shoot cultures maintained in an incubator, eight weeks after culture.

Growth variables	NO_3^-:NH_4^+ ratio		
	1:0	3:1	
	(Mean ± SE)	(Mean ± SE)	P[z]
Number of shoots<1 cm	2.80 ± 0.70	2.70 ± 0.76	0.92
Number of shoots≥1 cm	1.10 ± 0.28	3.30 ± 2.58	0.03
Total number of shoots	2.49 ± 0.61	7.54 ± 1.83	0.02
Average shoot length	1.88 ± 0.38	2.05 ± 0.32	0.73
Number small leaves(2-4 mm width)	0.22 ± 0.15	2.80 ± 0.79	0.01
Number of expanding leaves (>4 mm width)	2.67 ± 1.18	4.30 ± 1.26	0.36
Total number of leaves	2.89 ± 1.30	7.10 ± 1.80	0.07

[z] Mean separation by standard t-test

Effect of N Concentration on Shoot Growth Responses in an Incubator after 8 Weeks

No increase in average shoot length was detected as nitrogen levels increased from 20 to 60 mM ($P > 0.05$). The average length of the shoots ≥ 1 cm increased linearly with increasing total nitrogen concentration, even though the r^2 value was low (0.14) (Figure 6-3 A). There were positive curvelinear correlations between number of shoots ≥1 cm, total

shoot length, total number of leaves, number of expanding leaves and nitrogen concentrations (Figure 6-3 B–E). The nitrogen concentration of 40 mM was optimum for shoot culture growth with respect to variables such as number of shoots ≥1 cm, total shoot length, total number leaves and number of expanding leaves.

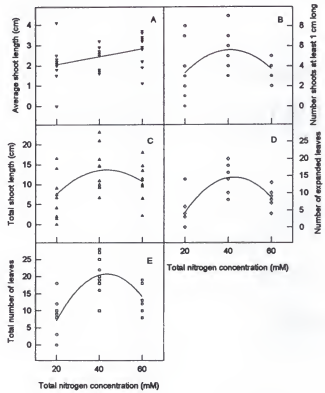

Figure 6-3. *In vitro* growth of 'Guaram 13' avocado shoots in response to varying concentrations of nitrogen (N) in the form of 3 NO_3^- : 1 NH_4^+. The growth variables and their regression lines are as follows: A) number of shoots≥1 cm, $Y = -3.2 + 0.43\ N + 0.005\ N^2$, $r^2 = 0.22$; B) average shoot length, $Y = 1.68 + 0.02\ N$, $r^2 = 0.14$; C) total shoot length, $Y = -7.45 + 0.97\ N + 0.61\ N^2$, $r^2 = 0.20$; D) number of expanding leaves, $Y = -27.1 + 1.7\ N + 0.02\ N^2$, $r^2 = 0.57$; E) total number of leaves, $Y = -5.8 + 2.1\ N + 0.002\ N^2$, $r^2 = 0.56$.

Effects of Atmospheric CO_2 and NO_3^-:NH_4^+ Ratio on Shoot Growth and Photosynthesis after 10 Weeks

There were no significant interactions between atmospheric CO_2 concentration and NO_3^-:NH_4^+ ratio for any of the growth variables measured. Shoot cultures grown on medium with a NO_3^-:NH_4^+ ratio of 3:1 had a significantly higher number of shoots ≥ 1 cm and a higher number of expanding leaves than those grown on medium with NO_3^-:NH_4^+ ratio of 1:0 (Table 6-2). The NO_3^-:NH_4^+ ratio had no effect on number of shoots < 1 cm, total number of shoots, average shoot length, number of small leaves and total number of leaves (Table 6-2). Cultures that were maintained in an elevated CO_2 environment produced significantly more shoots, shoots < 1 cm length (but not ≥ 1 cm length), and leaves (both small and expanding) than plants grown in an ambient CO_2 environment (Table 6-3); however, average shoot length was not significantly affected by the

Table 6-2. Effect of medium NO_3^-:NH_4^+ ratio at 20 mM on the growth variables and net photosynthesis of avocado shoot cultures after ten weeks in culture.

Growth variable	NO_3^-:NH_4^+ ratio		
	1:0	3:1	
	(Mean ± SE)	(Mean ± SE)	P[z]
Number of shoots<1 cm	5.00 ± 1.29	3.95 ± 0.60	0.4662
Number of shoots≥1 cm	2.60 ± 0.28	5.32 ± 0.98	0.0142
Total number of shoots	7.60 ± 1.36	9.26 ± 1.25	0.3736
Average shoot length	1.83 ± 0.12	2.13 ± 0.21	0.2255
Number small leaves(2-4 mm width)	10.30 ± 1.90	12.79 ± 3.60	0.5454
Number of expanding leaves (>4 mm width)	6.25 ± 1.14	12.89 ± 2.02	0.0078
Total number of leaves	16.55 ± 2.25	25.68 ± 4.61	0.0862
Net photosynthesis rate (μmol CO_2 m^{-2} s^{-1})	17.43 ± 4.58	13.98 ± 2.28	0.5120
Dry matter content (%)	16.26 ± 1.67	21.67 ± 3.94	0.2298

[z] Mean separation by standard t-test

Data across atmospheric CO_2 concentration treatments were pooled for the analysis, since their interaction with nitrogen ratio was not significant ($p>0.05$).

atmospheric CO_2 concentration (Table 6-2) or the medium NO_3^-:NH_4^+ ratio (Table 6-3). Net photosynthesis of the shoots was not affected by medium NO_3^-:NH_4^+ ratios (Table 6-2). Net photosynthesis of shoot cuttings from cultures grown in elevated CO_2 was significantly lower than that of cultures grown in ambient CO_2 (Table 6-3). The dry matter content was not significantly affected by atmospheric CO_2, NO_3^-:NH_4^+ ratios, or their interaction ($P > 0.05$); however, there was a tendency for shoot cultures grown in elevated CO_2 to accumulate more dry matter than those in an ambient CO_2 ($P < 0.06$) (Table 6-3).

Table 6-3. Effect of atmospheric CO_2 environment on the growth variables and net photosynthesis of avocado shoot cultures after ten weeks in culture[x].

Growth variable	Atmospheric CO_2 concentration		
	Elevated	Ambient	
	(Mean ± SE)	(Mean ± SE)	P[y]
Number of shoots<1 cm	6.05 ± 1.27	3.00 ± 0.57	0.0381
Number of shoots≥1 cm	4.74 ± 0.93	3.15 ± 0.53	0.1490
Total number of shoots	10.79 ± 1.50	6.15 ± 0.86	0.0121
Average shoot length	1.79 ± 0.10	2.16 ± 0.21	0.1296
Number small leaves(2-4 mm width)	17.16 ± 3.60	6.15 ± 0.81	0.0073
Number of expanding leaves (>4 mm width)	12.16 ± 1.87	6.95 ± 1.50	0.0370
Total number of leaves	29.32 ± 4.08	13.10 ± 2.14	0.0015
Net photosynthesis rate ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	10.61 ± 2.45	20.80 ± 3.89	0.0423
Dry matter content (%)	23.15 ± 3.92	14.77 ± 0.84	0.0637

[x] Mean separation by standard t-test

[y] Data across NO_3^-:NH_4^+ ratio were pooled for the analysis, since their interaction with atmospheric CO_2 concentration was not significant ($p>0.05$).

Effects of Atmospheric CO_2 and N Concentration on Shoot Growth Responses and Photosynthesis after 10 Weeks

After 10 weeks of culture, there were no interactions between nitrogen level and atmospheric CO_2 concentration for any of the variables measured ($P > 0.05$). Unlike observations 8 weeks after culture, nitrogen level had no significant effect on any variable measured 10 weeks after culture, except for dry matter content. Shoot dry matter content was negatively and curvelinearly related to nitrogen concentration, although the r^2 value was low (0.18) (Figure 6-4). Cultures maintained in an elevated CO_2 concentration had higher values for all growth variables measured than those maintained in an ambient CO_2 concentration, except for average shoot length (Table 6-4) (Figure 6-5). Net photosynthesis of shoots removed from cultures maintained in elevated atmospheric CO_2 was significantly lower than with shoots maintained in an ambient atmospheric CO_2 environment (11.1 ± 1.8 versus 16.5 ± 1.6 μmol CO_2 m^{-2} s^{-1}) (Table 6-4).

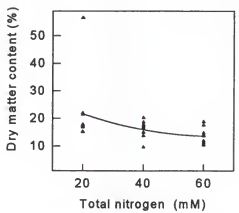

Figure 6-4. Dry matter content of avocado shoots *in vitro* in response to varying concentration of total nitrogen (N) in the form of 3 NO_3^- : 1 NH_4^+, after 10 weeks in culture. The regression line is represented with $Y = 30.8 - 0.54\ N + 0.0043\ N^2$, $r^2 = 0.18$. Data across treatments of atmospheric CO_2 concentrations were pooled for the analysis since their interaction with total nitrogen concentration were not significant ($P > 0.05$).

The Net Photosynthesis of Proliferating Shoot Cultures, Microcuttings with Subtended Callus and Plantlets and Their Plantlet-Derived Shoot Cuttings

As determined in a preliminary experiment, intact proliferating shoot cultures had negative net photosynthesis, indicating respiration, regardless of the atmospheric CO_2 concentration. Shoot cuttings that were removed from proliferating shoot cultures, however, had positive net photosynthesis value. The calli from the shoot cultures had high net respiration rates (Table 6-5). The net photosynthetic rate of plantlets grown in an elevated CO_2 environment was not significantly different from that of the plantlet-derived shoot cuttings ($P > 0.05$) (Table 6-5), indicating no significant effect of cutting on their net photosynthesis.

Table 6-4. The effect of atmospheric CO_2 concentration on the growth variables and net photosynthetic rate of avocado shoot cultures after ten weeks in culture[y].

Growth Variable	Atmospheric CO_2 concentration		P[z]
	Elevated	Ambient	
	(Mean ± SE)	(Mean ± SE)	
Number of shoots<1 cm	4.41 ± 0.59	3.07 ± 0.44	0.0734
Number of shoots≥1 cm	6.41 ± 0.70	4.53 ± 0.42	0.0259
Total number of shoots	10.83 ± 0.98	7.60 ± 0.66	0.0090
Average shoot length	2.31 ± 0.22	2.70 ± 0.17	0.1562
Number small leaves(2-4 mm width)	15.52 ± 2.43	9.28 ± 0.95	0.0219
Number of expanding leaves (>4 mm width)	20.38 ± 1.80	12.20 ± 1.33	0.0006
Total number of leaves	35.90 ± 3.27	21.48 ± 2.12	0.0006
Net photosynthesis rate (μmol CO_2 m^{-2} s^{-1})	11.13 ± 1.84	16.51 ± 1.60	0.0358
Dry matter content (%)	19.16 ± 2.82	15.08 ± 0.66	0.1790

[y] Data across total nitrogen concentration were pooled for this analysis since their interaction with atmospheric CO_2 concentration was not significant ($p>0.05$).
[z] Mean separation by standard t-test

A B C D E F G H

Figure 6-5. Shoot proliferation of 'Guaram 13' avocado on media of different N content under elevated and ambient CO_2 concentration, after ten weeks. Interveinal chlorosis is apparent on leaves of shoots cultured under elevated CO_2 concentration. A-D, shoot cultured under elevated CO_2 and E-H, shoot under ambient CO_2 environment. A and E, at 20 mM N of 1 NO_3^- : 0 NH_4^+. B and F, at 20 mM N; C and G at 30 mM N; D and H, at 40 mM N with ratio of 3 NO_3^- : 1 NH_4^+.

1 2 3 4 5 6 7 8 9 10

Elevated CO_2 Ambient

Figure 6-6. Plantlets of 'Guaram 13' avocado grown under elevated and ambient CO_2 concentration, after nine weeks. The first 5 plantlets from left were cultured under elevated CO_2 concentration; the last five plantlets were cultured under ambient CO_2 concentration. Interveinal chlorosis is apparent on leaves of the plantlets cultured under elevated CO_2 concentration.

Table 6-5 Net photosynthesis (μmol CO_2 g^{-2} s^{-1}) of intact avocado proliferating shoot cultures, their subcultured microcuttings and subtended callus in two atmospheric CO_2 concentrations, and intact plantlets and plantlet-derived shoots in an elevated CO_2 concentration before and after cutting[z].

Type of culture	Atmospheric CO_2 concentration	Intact culture	Subcultured Shoot cutting	Callus
		(Mean ± SE)	(Mean ± SE)	(Mean ± SE)
Shoot	Elevated	-0.20 ± 0.14	0.15 ± 0.07	-0.18 ± 0.05
	Ambient	-0.27 ± 0.24	0.38 ± 0.13	-0.21 ± 0.02
Plantlet	Elevated	0.40 ± 0.09	0.39 ± 0.09	

[z] Data represent means ± SEs of 4 replicates, after 10 weeks of culture.

Effect of Growth Environment on Plantlet Development 9 Weeks after Culture

Roots were visible from shoots after 3 weeks. After 9 weeks of culture, 90% of the shoots developed roots. In some of the cultures, callus also developed at the base of the shoot cuttings. Plantlets that developed in an elevated CO_2 environment grew more vigorously than those in the ambient CO_2 environment; they had thicker and longer stems, more and thicker roots and more leaves, although the older expanded leaves developed interveinal chlorosis (Figure 6-6). Shoot length and total leaf area of plantlets that developed in elevated CO_2 concentration were significantly greater than of plantlets in ambient CO_2 (Table 6-6). Number of leaves, average leaf area, number of roots, average root length and dry matter content were not significantly affected by atmospheric CO_2 concentration; however, root, stem, callus and leaf dry weights were significantly higher for plantlets that developed in an elevated atmospheric CO_2 environment than in a non-elevated CO_2 environment.

The net photosynthetic rate on a leaf area basis of plantlets grown in an elevated CO_2 environment (19.3 ± 4.8 μmol CO_2 m^{-2} s^{-1}) tended to be lower than that of plantlets in

an ambient CO_2 environment (31.3 ± 7.24 μmol CO_2 m^{-2} s^{-1}), when measured in an ambient atmospheric CO_2 environment (Table 6-6).

Table 6-6 'Guaram 13' avocado plantlet development in two atmospheric CO_2 environments, nine weeks after culture.

Growth variable	Atmospheric CO_2 concentration		
	Elevated	Ambient	
	(Mean ± SE)	(Mean ± SE)	P[z]
Shoot length (cm)	4.13 ± 0.28	2.74 ± 0.21	0.0008
Number of leaves	12.75 ± 0.80	9.54 ± 1.67	0.1048
Leaf area total (cm^2)	13.76 ± 1.76	6.88 ± 1.01	0.0034
Average leaf area (cm^2)	1.09 ± 0.14	0.93 ± 0.18	0.4595
Number of root	6.0 ± 0.65	4.64 ± 0.62	0.1449
Average root length (cm)	4.74 ± 0.42	4.42 ± 0.54	0.6437
Root dry weight (g)	0.02 ± 0.00	0.01 ± 0.00	0.0062
Stem dry weight (g)	0.02 ± 0.00	0.01 ± 0.00	0.0148
Callus dry weight (g)	0.05 ± 0.01	0.03 ± 0.01	0.0150
Leaf dry weight (g)	0.07 ± 0.01	0.03 ± 0.00	0.0011
Net photosynthesis rate (μmol CO_2 m^{-2} s^{-1})	19.34 ± 4.84	31.35 ± 7.24	0.1900
Dry matter content (%)	18.84 ± 3.65	16.94 ± 2.67	0.2849

[z] Mean separation by standard t-test

Discussion

Murashige & Skoog (1962) salt-based media have been successfully employed for *in vitro* propagation of plants, including herbaceous plants such as tropical foliage (Miller & Murashige, 1976), vegetable crops (Seckinger, 1991), woody plants, i.e., *Rosa* spp. (Ma et al., 1996) and tropical and subtropical fruit trees (Litz & Jaiswal, 1991) that are mostly woody perennials. MS-based media have been shown to be unsatisfactory for shoot culture of some woody species such as *Dipterocarpus intricatus* (Linington, 1991), *Pistachia vera* (Barghchi & Alderson, 1985), *Kalmia latifolia* (Lloyd & McCown, 1980),

and avocado (Pliego-Alfaro et al., 1987). Therefore, several modifications of MS formulation have been attempted. Reduction of the major and or minor salts concentration to 30–50% of the original concentration improved rooting of juvenile avocado shoots (Pliego-Alfaro, 1988), but not the multiplication of adult shoot cultures (Pliego-Alfaro et al.., 1987). Omission of NH_4NO_3 from the standard medium formulation was reported to improve the survival rate of juvenile phase shoot cultures to 100%, although the multiplication rate was still limited (Witjaksono, 1991).

In the current study, the multiplication rate of avocado shoot cultures was not enhanced by increasing the KNO_3 concentration as the sole inorganic nitrogen source. Shoot necrosis, leaf abscission and reduction of leaf and shoot production were observed with KNO_3 concentrations higher than 20 mM; however, incorporation of 25% inorganic ammonium to total inorganic nitrogen in the medium significantly improved the multiplication rate and the health of the cultures as indicated by the production of healthier and more leaves.

The optimum nitrogen concentration for shoot multiplication, determined at 8 weeks after culture, was 40 mM with a 3:1 NO_3^-:NH_4^+ ratio. The theoretical multiplication rate under these conditions would be 13-fold for each 8 week subculture cycle, and 100,000 plantlets could be theoretically produced from one nodal stem in one year. This multiplication rate is higher than the multiplication rate of 100 fold per 4 month subculture cycle that was reported for *P. indica* (Nel et al.., 1982), 3-fold per month cycle for 'Hass' and 'Hopkin' non clonal avocado (Cooper, 1987) and 77,000 plants per year for *P. palustris* (Kane et al.., 1989).

The shoot proliferation occurs by axillary branching. This is consistent with previously reported results for avocado (Schall, 1987; Cooper, 1987), *P. schiedaena* (Gonzales-Rosas et al.., 1985), *P. palustris* (Kane et al.., 1989) and *P. indica* (Nel et al.., 1985; Campos & Pais, 1996).

Due to the high respiration rate of the basal callus in avocado shoot cultures, intact proliferating shoot cultures show net positive respiration, although subcultured shoots show net positive photosynthesis. Net photosynthesis was measured using shoots that were separated from callus in proliferating shoot cultures. This was justified since dissection of the plantlets did not significantly affect net photosynthesis of the dissected shoots.

Growing avocado shoot cultures or plantlets in an elevated CO_2 concentration resulted in higher biomass production as indicated by a greater number of total leaves and number of shoots and a greater plant dry weight than cultures grown in an ambient CO_2 environment. Greater biomass production due to higher CO_2 levels was also reported for *in vitro* plantlets of banana (Navarro et al.., 1994), potato (*Solanum tuberosum*) (Kozai et al.., 1988a), *Cymbidium* (Kozai et al.., 1988b), carnation (*Dianthus caryophyllus* L) (Kozai & Iwanami, 1988), strawberry (*Fragaria* x *ananassa* Duch), raspberry *Rubus idaeus* L.) and asparagus (*Asparagus officinalis* L.) (Laforge et al., 1991). Similar results were also found with *ex vitro* studies of banana (Schaffer et al., 1996), mangosteen (*Garcinia mangostana* L.) (Downton et al., 1991), acclimatized plantlets of strawberry (Desjardins et al., 1987) and acclimatized *in vitro* propagated, non-rooted grape shoots (Lakso et al., 1986).

Net photosynthesis of avocado shoots maintained in a growth chamber without CO_2 enrichment is in the range that has been observed under field conditions for avocado (14–20 μmol CO_2 m^{-2} s^{-1}) (Whiley & Schaffer, 1994); however, the net photosynthetic rates (31 ± 7 μmol CO_2 m^{-2} s^{-1}) of plantlets grown in an ambient CO_2 environment were considerably higher than those reported in the literature. Consistent with other experiments, long-term exposure to an elevated CO_2 environment significantly reduced net photosynthesis of the avocado shoots and plantlets. For example, Navarro et al. (1994) found that photosynthetic fixation of *in vitro*-grown banana plants at ambient CO_2 concentration decreased as CO_2 concentration of the growth environment increased.

Similar results were also reported by Schaffer et al. (1996) working with banana in an environmentally controlled glasshouse.

The decrease in photosynthetic efficiency of plants grown in an elevated CO_2 atmosphere does not explain the high biomass production under such conditions. Several studies have demonstrated that the actual photosynthetic rates of plants adapted to high CO_2, when measured in their elevated CO_2 environment, were higher than the rate of plants grown at ambient CO_2 concentration (Dube & Vidaver, 1992; Kozai & Iwanami, 1988; Kozai et al., 1988a; Kozai et al., 1988b). Schaffer et al. (1996) demonstrated that net photosynthesis of banana plants increased with increasing CO_2 concentration in the cuvettes, but plants that were exposed to elevated CO_2 were less photosynthetically efficient than those exposed to ambient CO_2 environment. The higher actual photosynthetic rate in an elevated CO_2 environment was due to increased CO_2 in the environment available for fixation (Schaffer et al., 1996). The higher actual photosynthetic rates in the elevated CO_2 environment presumably resulted in the increased biomass for those plants compared to plants grown at ambient CO_2 (Schaffer et al., 1996).

As a summary, a protocol for enhancing multiplication of healthy shoot cultures of juvenile phase 'Guaram 13' avocado has been developed. Avocado shoots and plantlets *in vitro* are photosynthetically active regardless of the availability of sucrose in the medium. Under an elevated compared to a non-elevated CO_2 environment, the mixotrophic avocado shoots and plantlets grew better, even though their photosynthetic efficiencies were lower. Plantlets (i.e., rooted shoots) have higher photosynthetic efficiencies than do shoot culture-derived shoot cuttings *in vitro*. The practical implications of these observations are as follows: 1) plantlets developed under elevated CO_2 would provide vigorous planting materials with photosynthetic efficiency comparable to plants *ex vitro*; 2) shoot cuttings from *in vitro* shoot cultures are photosynthetically active, indicating the potential for combining acclimatization and rooting *ex vitro*; however a rooting step *in*

vitro would be a better method for acclimatization since plantlets have higher photosynthesis efficiencies than shoot culture-derived shoot cuttings.

CHAPTER 7
PROTOPLAST FUSION BETWEEN AVOCADO AND ITS RELATIVES, INCLUDING *NECTANDRA CORIACEA* AND *PERSEA SPP.*

Introduction

The major production problem of avocado is root-rot disease caused by *Phytophthora cinnamomi*. This avocado disease causes serious damage worldwide, especially in areas with heavy soil and poor drainage (Gustafson, 1976; Whiley, 1992). In California alone the losses due to this disease have been estimated to be $6,000,000 annually (Coffey, 1987). Breeding rootstock with resistance to this disease has been slow due to the absence of resistance traits in *Persea americana* and other sexually compatible species in the subgenus *Persea* (Pliego-Alfaro & Bergh, 1992). Resistance traits, however, are available in most of the small-fruited *Persea* species within the subgenus *Eriodaphne* of the genus *Persea* (Kopp, 1966), e.g., *P. borbonia*, *P. cinerascens*, *P. pachypoda*, etc. and in other related genera of the Lauraceae, i.e., *Nectandra*, *Phoebe* and *Ocotea* (Zentmeyer, 1980). Unfortunately, these resistant small seeded species are both sexually and graft incompatible with *Persea americana* and other large-fruited *Persea* spp. in the subgenus *Persea* (Bergh, 1975; Pliego-Alfaro & Bergh, 1992; Bergh & Lahav, 1996). An alternative way that has been proposed to bridge the incompatibility barrier is by protoplast fusion (Bergh & Ellstrand, 1986; Pliego-Alfaro & Bergh, 1992).

Protoplast fusion has been successfully used to produce somatic hybrids with pest and disease resistance and stress tolerance characteristics of one of the parents and include potato and tomato, *Brassica*, eggplant and tobacco and their relatives (summarized in Bajaj, 1994). The somatic hybrids produced from the fusion of eggplant with its distant relatives grew poorly due to poor rooting. The plants were also highly sterile; however,

somatic hybrids produced from eggplant with its more closely related species such as *S. aethiopicum* showed vigorous growth and high yields (Sihachakr et al., 1994). This indicates that somatic hybrids involving distantly related species may not have direct utility, although they can be used in breeding programs.

The potential of somatic hybridization for fruit crop improvement has been demonstrated largely with citrus (Grosser & Gmitter, 1990; Louzada & Grosser, 1994) and with pear (Ochatt et al., 1989) and passion fruit (Otoni et al., 1995; Dornelas et al., 1995). Somatic hybrid plants have been obtained following protoplast fusion between sexually and graft incompatible 'Colt' cherry (*Prunus avium x pseudocerasus)* and wild pear (*Pyrus communis*) (Ochatt et al, 1989). The hybrids were graft compatible to both parents (Ochatt & Patat-Ochatt, 1994). Somatic hybrid plants from sexually incompatible genera of Rutaceae species, i.e., between *C. sinensis* and *Atalantia ceylanica* (Louzada et al., 1993) and between *C. sinensis* and *Severinia disticha* (Grosser et al., 1988) have been reported. Somatic hybrids among Rutaceae species that are sexually incompatible and difficult to graft onto each other, i.e., *C. sinensis* with *Clausena lansium* (Lour.) Skeels has also been obtained, but it did not survive (Louzada & Grosser, 1994).

The purpose of this work was to develop a protocol for protoplast fusion between avocado and the root-rot resistant *Nectandra* and small-fruited *Persea* species.

Materials & Methods

Plant Materials

Nectandra coriacea mature fruits as indicated by the dark skin color were collected from Everglades area. The fleshy part of the fruits (carpels) were removed and the seeds were washed thoroughly with running tap water. The seeds were air dried, placed in plastic bags and stored in a refrigerator until use. Mature seeds of *P. borbonia*,

P. pachypoda and *P. cinerascens* were collected from the South Coast Experiment Station of the University of California and were supplied by Dr. John A. Menge of the University of California, Riverside. They were stored in a refrigerator until use. All these species were used as sources of leaf mesophyll protoplasts.

Seeds were germinated *in vitro*. The seed coats were removed and the embryos were washed with tap water to remove any seed coat remnants. They were surface disinfested in a 20% (v/v) solution of commercial bleach containing 10–20 drops l^{-1} of Tween 20® for 10–20 min and rinsed with 2 changes of sterile, deionized water. The embryos were cultured on a basal plant growth medium consisting of MS salts, 0.4 mg l^{-1} thiamin HCl, 100 mg l^{-1} myo inositol, 30 g l^{-1} sucrose and solidified with 2 g l^{-1} Gel Gro gellan gum. The pH of the medium was adjusted to 5.7–5.8 with KOH or NaOH prior to addition of gelling agent. The medium was dispensed in 100 x 15 mm sterile plastic Petri dishes in 20 ml aliquots. There were 8–12 embryos per Petri dish.. The Petri dishes were sealed with Parafilm® and maintained in darkness at 25°C.

Seeds began to germinate after 3-5 days. After 10–15 days, seedlings had ≥ 2 cm long roots and ≥ 0.5 cm long shoots were transferred individually onto filter bridges in liquid plant growth medium contained in test tubes. Plant growth medium consisted of MS salts without NH_4NO_3, 1 mg l^{-1} thiamine-HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and 400 mg l^{-1} glutamine. The pH was adjusted to 5.7–5.8 and medium was distributed in 20 ml quantities to 25 x 150 mm test tubes, each of which contained a filter paper support (Whatman # 2). The tubes were capped with kaputs (polypropylene closures) and autoclaved at 1.1 kg cm^{-2} and 121°C for 15 min. After culturing the seedlings into the nutrient tubes, the kaputs were replaced with Suncaps® (Sigma Chemical, St. Louis, MO). The cultures were maintained under irradiance of 50–60 µmol photon m^{-2} s^{-1} for 16 h. The liquid medium was replaced at 2 month intervals at which time the leaves were removed for protoplast isolation and the shoots were cut back to ca. 0.5 cm above the cotyledons.

Protoplast Isolation

Young, non-expanded leaves of 0.3–1 cm width usually were removed from the *in vitro*-grown seedlings and placed in liquid medium-wetted sterile glass Petri dishes. There were usually 1–3 leaves that could be extracted from each seedling and usually 20–30 seedlings were necessary to yield one gram of leaf tissue. The leaves were feathered with a sharp blade parallel to the midvein. The leaf tissue (ca. 500 mg) was transferred into a 50 ml Erlenmeyer flask containing 5 ml enzyme mixture (Table A-4) and 6 ml 0.7 M MS⁻ 8P (Table A-2) and secured with aluminum foil and Nescofilm. The digestion mixture was incubated in darkness at 25° C for 16 h on a rotary shaker at 75–100 rpm. The protoplasts were purified as described (see Chapter 5; Grosser & Gmitter, 1990). Embryogenic cultures of 'T362', 'Thomas' zygotic embryo-derived culture and 'Hass' that was no longer embryogenic were used as a source of avocado protoplasts. Avocado protoplasts were produced as described in Chapter 5. The resulting protoplasts were resuspended in 0.7 MS⁻8P at 20 X their pelleted volume.

Protoplast Fusion

Protoplast fusions were performed using the PEG method (Grosser & Gmitter, 1990). Avocado protoplasts and leaf mesophyll protoplasts were mixed in a ratio of 1:2 volume which yielded a protoplast ratio of ca. 1:4. Protoplast fusion was induced in sterile plastic Petri dishes (60 x 15 mm). Into each fusion dish, 2 drops of protoplast mixture were added, and 2 drops of PEG solution (Table A-6) were added immediately to the center of the droplets. Twelve min later, two drops of elution solution (Solution A + B with A:B ratio of 9:1) were added, with one drop at opposite sides of the liquid pool containing the aggregated protoplast mixture and PEG. After another 12 min, 12–15 drops of fresh 0.7 M MS⁻8P medium were added to the periphery of the liquid droplet containing fusing protoplasts. After 5 min, the PEG, elution solution and the medium that

formed the droplet were removed carefully with a Pasteur pipette and immediately replaced with 12–15 drops of fresh 0.7 M MS⁻8P medium. This washing step was done carefully 3 X with 10 min intervals so as not to remove protoplasts in the center of the dish.

Two sets of fusion experiments were done: 1) between zygotic-derived embryogenic 'T362' avocado protoplasts and leaf mesophyll protoplasts of *Nectandra coriacea* and between embryogenic nucellar-derived 'Thomas' protoplasts and leaf mesophyll protoplasts of *P. borbonia*; 2) between embryogenic protoplasts of either 'Thomas' zygotic embryo-derived cultures or 'Hass' that had lost embryogenic potential with leaf mesophyll protoplasts of *P. pachypoda*. These fusion combinations were: 'Hass' + *P. pachypoda*, 'Hass' + 'Thomas' zygotic, 'Thomas' zygotic + *P. pachypoda*, and mixture of protoplasts without fusion of 'Hass' and 'Thomas' zygotic, 'Thomas' zygotic and *P. pachypoda*, and individual protoplasts of 'Hass', 'Thomas' zygotic and *P. pachypoda*.

Protoplast culture

Protoplasts were cultured directly in the fusion dish. After the final wash, 3 ml of 0.4 M MS⁻8P medium were added to each Petri dish and was carefully spread to form a shallow liquid cover over the entire Petri dish. The dishes were sealed with Nescofilm® and incubated in darkness at 25°C. After 2–5 weeks by which time microcallus or proembryonic masses of 100–200 µm had formed, the cultures were subcultured at a diluted density of 20–40-fold in 3 ml of 0.15 M MS⁻8P medium. After 1 month more, somatic embryos and proembryonic masses of 1–2 mm diameter were visible. These somatic embryos and proembryonic masses were transferred to semisolid somatic embryo development medium.

For fusions involving cultures that had lost their embryogenic potential, ca. 6–8 dishes were cultured for each fusion treatment. Subculture to a lower density in medium with lower osmoticum or the same osmoticum was conducted 40 days after fusion due to the slow growth of the cultures. For cultures that formed microcalli or proembryonic masses prolifically, they were subcultured with densities of 6 to 20-fold dilution. For cultures that did not grow vigorously, several culture dishes were pooled before replacement with fresh medium.

For culture volume determination, 3 dishes representing prolific cultures and 6 to 8 cultures from non-prolific cultures were randomly selected. Sampling of proembryonic masses for DNA analysis was done by randomly selecting 2–3 dishes (at the subculture 40 days after fusion) from each dish. The proembryonic masses were pooled for each fusion combination for DNA isolation.

Somatic Embryo Development and Plant Regeneration

Details of the method for stimulating somatic embryo development and plant regeneration from fusion experiment are described in Chapter 4. Somatic embryos and proembryonic masses of 1–2 mm diameter were used as inocula for somatic embryo development.

Hybrid Confirmation Using RAPD

Sampling of Culture for PCR-RAPD Analysis

From the fusion experiment involving and *N. coriacea*, one somatic embryo converted into a plantlet that were subsequently micropropagated. Leaves from this materials were sampled for RAPD analysis and compared with leaves sampled from shoot cultures that originated from somatic embryo developed from zygotic-derived 'T362' avocado embryogenic cultures. Leaves of *in vitro* seedling of *N. coriacea* and protoplast-

derived proembryonic masses of zygotic-derived 'T362' were sampled and served as parents for the putative hybrid. Proembryonic masses that developed from fusion between nucellar-derived 'Thomas' and *P. borbonia* protoplasts were sampled ca. 1 month after subculture in diluted density. From the second fusion experiment, proembryonic masses that developed after subculture in diluted density and lower osmoticum were sampled.

DNA Isolation

DNA was isolated using the SDS method modified according to Mourão-Filho (1995). Tissues consisting of leaves, proembryonic masses or somatic embryos, each ca. 100 mg, were ground in liquid nitrogen in sterile 1.5 ml Eppendorf tubes using forceps. Extraction buffer containing 100 mM Tris-HCl pH 8.0, 50 mM Na_2EDTA (pH 8.0), 500 mM NaCl, 10 mM β mercaptoehtanol and 3% SDS were added to the samples in 700 µl quantity and the mixture was macerated for a few more min. After all samples were prepared, the tubes were incubated for 10 min at 65° C in a thermocycler (PTC 100, Programmable Thermo Cycler, MJ Research Inc.). The samples were then centrifuged for 10 min at 12,000 rpm. The supernatants were transferred to clean tubes and to each tube an equal vol of phenol was added and then vortexed for a few sec and then centrifuged at 12,000 rpm for 10 min. The aqueous, upper phase was transferred to a clean tube and an equal vol of chloroform:isoamyl alcohol (24:1) was added. The tubes were vortexed for a few sec and centrifuged at 12000 rpm for 10 min. The aqueous phase was transferred to a clean tube and mixed with 0.6 vol of isopropanol and 0.1 vol of 7.5 M ammonium acetate to precipitate the DNA. The tubes were inverted back and forth very gently for 2 –3 min and then centrifuged at 12000 rpm for 5 min. The supernatant was decanted and the pellet was washed with 500 µl 70% ethanol and then centrifuged at 12000 rpm for 2 min. The ethanol was decanted and the tubes were blotted carefully with Kimwipes and then dried at 65° C in a thermocycler for 5 min. The pellets were dissolved in 50 µl deionized, sterile

H_2O and stored at -20° C before use. The DNA concentration was quantified by spectrophotometry at $\lambda = 260$ nm, and was then diluted to ca. 50 ng μl^{-1}.

Alternatively, DNA was isolated from samples (derived from fusions involving avocado cultures that had lost embryogenic potential) using the Phytopure Plant DNA Isolation Kit from Nucleon Bioscience (a division of Scotlab Limited, United Kingdom) (Lee & Nicholson, 1997).

Polymerase Chain Reaction (PCR)-Random Amplified Polymorphic DNA (RAPD)

PCR-RAPD was performed using a DNA Thermal Cycler (Perkin Elmer Cetus) with the following DNA amplification program: 45 cycles of 94° C for 1 min, 36° C for 1 min, 72.2° C for 1 min and followed by soak at 4° C. Each PCR tube contained 25 µl reaction mixture consisting of 15.15 µl H_2O, Stouffel buffer containing 100 mM Tris-HCl, 100 mM KCl, pH 8.3 (Perkin Elmer), 25 mM $MgCl_2$, 1 unit Ampli® Taq polymerase (Stouffel fragment), 400 µM dNTPs, primer and 10–50 ng genomic DNA. The primers tested included A18, F13 and J16 (Operon Technology, Alameda, CA)

The DNA was separated in 2% agarose gel electrophoresis in TEA buffer with electrical potential of 200 mV for 5 min followed by 160 mV for 80 min. The gel was stained with 5 ppm ethidium bromide for 30 min for observation and photography using UV light.

Results

Protoplast Isolation from Leaf Tissue

High protoplast yields were obtained from dark green, young and incompletely expanded leaves of *Nectandra coriacea* (Table 7-1). Protoplasts from this preparation were small, with ca. 10–20 µm diameter. Protoplasts were released only when the leaf tissue was agitated at 100 rpm.

Persea species generally did not provide good yields of mesophyll protoplasts; however, good yields of *P. pachypoda* protoplasts were obtained when soft and tender expanded leaves were digested. The protoplasts of *P. pachypoda* were ca. 20 –30 µm diameter. *Persea borbonia* and *P. cinerascens* provided low yields, i.e., ca. 0.02 ml pelleted protoplasts (ca. 1–3 x 10^5 protoplasts per gram fresh tissue). *Persea cinerascens* protoplasts were released prolifically during enzyme incubation, but only a few protoplasts could be recovered after centrifugation.

Table 7-1. Leaf mesophyll protoplast yield from *in vitro* seedlings of *Nectandra* and *Persea* sp.

Species	No. Trials	Yields
Nectandra coriacea	3	1.3 x 10^7 protoplast g^{-1}
Persea borbonia	3	low, ca. 0.02 ml pellets
Persea pachypoda	2	3 x 10^6
Persea cinerascens	3	low, ca. 0.02 ml pellets

Fusion between Avocado 'T362' and *Nectandra coriacea* and 'Thomas' Nucellar

Initial fusion experiments involving equal volume of mesophyll protoplasts of 'T362' avocado and *N. coriacea* resulted in fusion frequencies of 1–2%. Alternatively, protoplasts were mixed in a proportion of 1:2 vol of avocado: *N. coriacea* prior to fusion. In this proportion, the fusion frequency reached 10–20%. The fused protoplasts could be identified by the presence of chloroplasts and starch granules (Figure 7-1 A).

Approximately 600 somatic embryos developed from 4 fusion attempts between avocado 'T362' and *Nectandra coriacea*. Some somatic embryos (ca. 3–5 mm diameter) germinated precociously, and did not form shoots. Most of the embryos that developed

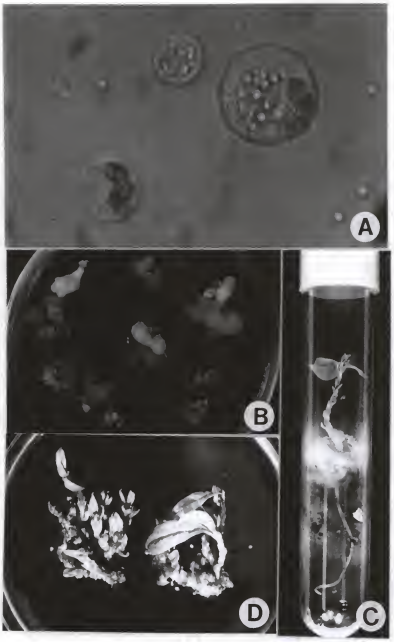

Figure 7-1. Protoplast fusion between zygotic embryo-derived 'T362' avocado and *Nectandra coriacea*, the somatic embryos, plantlet regeneration and shoot proliferation. (A) A heterokaryon. Note the chloroplasts (dark) and starch granules (white) intermixed in the fused protoplast. (B) Large somatic embryos from fusion experiment. (C) A plantlet regenerated from a fusion experiment. (D) Proliferating shoots of D.

were larger (1.5-2 cm diameter) (Figure 7-1 B) than the somatic embryos that were regenerated from protoplasts without fusion (Figure 5-11 B). Some somatic embryos formed secondary proembryonic masses at their bases. Approximately 11 months after fusion, one somatic embryo converted successfully into a plantlet (Figure 7-1 C).

The shoot was removed and proliferated on ASP medium (Table A-1). On this medium, the nodal stem segments and shoot tips produced multiple shoots that did not elongate. Shoot elongation and leaf expansion were obtained after transfer of shoots from medium with 4.44 μM BA to medium with 0.4 μM BA (Figure 7-1 D). The shoots produced only a few roots with 20% rooting frequency after 8 to 12 weeks and this was accompanied by massive callus formation at the base of the shoot cuttings. The plantlets did not survive acclimatization. RAPD analysis of this putative hybrid shoots did not confirm a somatic hybrid origin (Figure 7-2).

RAPD analysis of proembryonic masses that developed from fusion between nucellar-derived 'Thomas' avocado with *P. borbonia* protoplasts did not confirm somatic hybrid origin (data not shown).

Fusion between Avocado with either avocado or *P. pachypoda*

The growth and development of protoplasts from different sources were different. The leaf mesophyll protoplasts of *P. pachypoda* did not divide and became necrotic and disintegrated after 40 days. Protoplasts derived from 'Thomas' zygotic budded but did not divide, whereas 'Hass' protoplasts formed microcalli. When the protoplasts were mixed without fusion, they behaved in the same manner. When the protoplasts were fused, there was improvement of their growth and differentiation. The fused 'Thomas' (with *P. pachypoda*) protoplasts formed microcalli, while 'Thomas' zygotic derived and *P. pachypoda* protoplasts without fusion failed to divide. Similarly, fusion of 'Hass' with either *P. pachypoda* or 'Thomas' zygotic-derived protoplasts resulted in development of

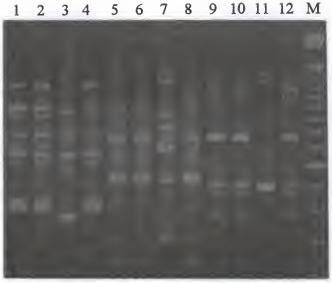

Figure 7-2. RAPD banding patterns of leaf from a somatic embryo that developed from protoplasts fusion between embryogenic cultures of a zygotic-derived avocado line 'T362' and *Nectandra coriacea*, and its parental sources. Lane 1, 5 and 9 = leaves of shoots derived from a somatic embryo from zygotic-derived 'T362'; lane 2 6 and 10 = leaves of shoot derived from a somatic embryo from fusion between 'T362' and *N. coriacea*; lane 3, 7, 11 = leaves of *N. coriacea*; lane 4, 8 and 12 = protoplast-derived proembryonic masses of 'T362'. Lane 1–4 used primer A18; lane 5–8 used primer F13; and lane 9–12 used primer J16.

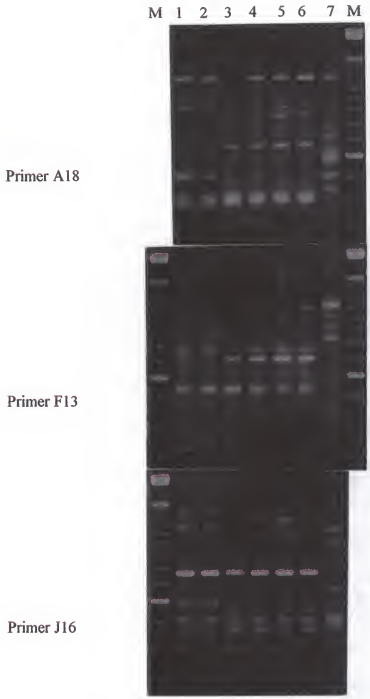

Figure 7-3. RAPD banding patterns of proembryonic masses of avocados and their fusion with other avocado and *P. pachypoda*. Lane M = marker, 1= zygotic 'Thomas', 2 = zygotic 'Thomas' + *P. pachypoda*, 3 = 'Hass' + zygotic 'Thomas' (at protoplasts/microcalli stage, before subculture at diluted densities), 4 = 'Hass' + zygotic 'Thomas', 5 = 'Hass', 6 = 'Hass' + *P. pachypoda*, 7 = *P. pachypoda*.

Table 7-2. Growth and development of protoplasts after fusion and subculture of microcalli/proembryonic masses following subculture in fresh medium.

Protoplast Source	Treatment	Culture Growth after 40 days			Culture Growth after Subculture Treatment for 30 days				RAPD pattern[b]
		Sedimented Vol. (ml)	Morphology: microcallus or PEM	Color	Dilution/ Osmolarity	No. PEM	Dilution/ Osmolarity	No. PEM	
'P. pachypoda'	no fusion	<0.003	no division	black	--	--	--	--	--
'Thomas zygotic' (Thomas)	no fusion	0.006	no division	black	--	--	--	--	--
'Hass'	no fusion	0.053	microcallus	yellow	13 x/0.15 M	0	10 x/0.4 M	0	--
'Thomas ', *'P. pachypoda'*	mix, no fusion	0.006	no division	black	--	--	--	--	--
'Thomas', 'Hass'	mix, no fusion	0.030	microcallus	blackish yellow	1 x/0.15 M	0	--		'Hass'
'Thomas' + *'P. pachypoda'*	fusion	0.005	microcallus	blackish	0.25 x/0.15 M	7	--	--	'Thomas'
'Hass' + *'P. pachypoda'*	fusion	0.083	microcallus, PEMs[a]	yellow	6-20 x/0.15 M	0	20 x/0.4 M	241	'Hass'
'Hass' + 'Thomas'	fusion	0.156	microcallus, PEMs	yellow	20 x/0.15 M	480	20 x/0.4 M	454	'Hass'

[a] PEM = proembryonic masses

[b] see Figure 7-3 for the RAPD banding patterns.

microcalli and proembryonic masses, whereas without fusion 'Hass' protoplasts developed only microcallus. These differences in growth and development were also reflected in the volumes of the cultures (Table 7-2).

Development following subculture at lower culture density in medium of lower osmolarity improved culturability following the fusion treatment. While no proembryonic masses could be recovered from 'Hass' protoplasts, proembryonic masses could be recovered following the fusion treatment. The proembryonic masses that developed after fusion showed differences according to fusion combination, i.e., 'Hass' + 'Thomas' zygotic resulted in the highest number of proembryonic masses followed by 'Hass' + *P. pachypoda*, and the lowest PEM development was with 'Thomas' zygotic + *P. pachypoda*.

RAPD analysis indicated no DNA complementation from proembryonic masses from the fusion experiments. DNA banding patterns resembled those of the most vigorous parents (Figure 7-3). For example, the DNA banding pattern of 'Thomas' zygotic + *P. pachypoda* fusion was that of 'Thomas', while DNA of 'Hass' + 'Thomas' zygotic was that of 'Hass' (Table 7-2).

Discussion

Protoplasts could be isolated from seedling leaves of *Nectandra* and *Persea* species that are resistant to Phytophthora root-rot. While the protoplast yields were considerably higher for *Nectandra*, the use of very young leaves required many seedlings and protoplast yields were not very high. The use of very young leaves may have caused the small size of the protoplasts. The isolation of protoplasts from the *Persea* species was more difficult and yields were inconsistent. The successful isolation of *Persea* leaf mesophyll protoplasts was dependent on appropriate source tissue and agitation of digestion mixtures that is higher than the standard 50 rpm reported by Grosser and

Gmitter, 1990; however, agitation at 125 rpm instead of 3–50 rpm was required to release protoplasts from leaves of *in vitro* propagated peach (*Prunus persica*) plants (Mills & Hammerschlag, 1994).

The shoots that were proliferated from somatic embryos derived from fusion experiments were not of hybrid origin. Nonetheless, the shoots showed increased sensitivity to BA. The ploidy of this material awaits confirmation. The difficulty to root of such shoots may not be related to protoplast fusion procedure but may be genotype dependent, since somatic embryo-derived shoots from the same embryogenic culture line also demonstrated similar rooting behavior.

The use of embryogenic cultures that have diminished their embryogenic potentials for one of the fusion parents in combination with a leaf parent has been employed successfully to produce somatic hybrids with citrus (Grosser & Gmitter, 1990). According to Grosser and Gmitter (1990) the process of fusion itself may trigger embryogenic competence. Using a similar approach, avocado cultures that had lost their embryogenic potentials were fused with leaf mesophyll protoplasts of *Persea spp.* No somatic embryos were recovered even though improvement of protoplast growth and differentiation were observed following fusion.

The failure to obtain somatic hybrids in these fusion experiments may not necessarily reflect incompatibility between the parents since large numbers of somatic embryos did not develop to maturity. Another limitation of this study is that the number of fusions was limited by the availability of non-avocado parent protoplasts. Alternative methods for isolating non-avocado protoplasts for fusion experiments, e.g., callus, suspension cultures, and leaves from continuously proliferating shoot cultures need to be explored. In addition, fusion should also be attempted for avocado with other species belonging to different genera in the family Lauraceae that have fruit size and shape similar to avocado such as *Endiandra* and *Beilschmieidia* (Schroeder, 1995). Borys et al. (1993) reported that pulp of *Beilschmieidia annay* (Blake) Kosterm., has taste and texture better

than or similar to avocado. Unfortunately, the graft and sexual compatibility of this species and its resistance to root-rot have not been studied conclusively (Schroeder, 1995). More studies involving fusion between avocado and related species need to be done in order to assess the viability of protoplast fusion for combining avocado with incompatible species.

CHAPTER 8
SUMMARY AND CONCLUSIONS

Summary

In this study, several *in vitro* protocols for avocado have been either improved or newly developed. These include initiation and establishment of embryogenic cultures of various genotypes and elite selections, somatic embryo development, shoot proliferation of juvenile materials, protoplast isolation, culture and regeneration and protoplast fusion between avocado and related species.

Embryogenic cultures have been induced from immature zygotic embryos from 12 avocado cultivars and from nucellar explants of four avocado cultivars. This is the first report of the recovery of embryogenic nucellar cultures of avocado. The strategy involved explanting onto a sterile, semisolid induction medium consisting of modified B5 macroelements, MS microelements and organics (Appendix 1), and which was supplemented with the plant growth regulator picloram. Embryogenic cultures, which consisted of proembryonic masses, were maintained either on semisolid or in liquid proliferation medium consisting of MS major salts and other addenda (MSP) (Appendix 1), and supplemented with picloram. This is the first report on establishment of embryogenic suspension cultures of avocado. Filter sterilization of the liquid growth medium significantly improved the growth of the culture.

Somatic embryo development was initiated using small proembryonic masses or singulated somatic embryos (diameter <1.8 mm) as an inoculum on maturation medium. Somatic embryo development was genotype-dependent and affected by osmolarity sucrose

and gelling agent concentration. The composition of optimized somatic embryo development medium was similar to the proliferation medium (without picloram) and solidified with 6 g/l Gel-Gro™ gellan gum. Somatic embryos developed from globular to heart to cotyledonary stage somatic embryos, which were white, opaque and round with diameter ca. 0.8–1.2 cm. After subculture of cotyledonary somatic embryos onto medium supplemented with 4.5 μM BA and solidified with gellan gum for 2–3 subcultures of 2–3 months duration, the somatic embryos were ca. 1–2 cm diameter. Mature somatic embryos were subcultured on germination medium which was similar to maturation medium in composition, but supplemented with 2.89 μM GA_3. After 8–12 months combined on maturation and germination medium, a low frequency (ca. 5%) of somatic embryos developed shoots with or without roots.

Somatic embryogenesis has also been initiated from nucellar explants of 'Thomas', 'Hass', 'Lamb' and 'T362' on the same induction medium. Somatic embryo maturation occurred only with 'Thomas'; however, although somatic embryos germinated, there was no shoot formation

Using 'Guaram 13' as a model, protocols for shoot proliferation of juvenile origin has been improved by manipulating the total nitrogen content and the ratio of NO_3^-:NH_4^+ of the plant growth medium. Nodal or shoot tip explants of 1 cm have a survival rate of 100% and proliferated at the rate of 12-fold at each 8 week interval when cultured on medium containing modified MS salts (nitrogen content was reduced to 40 mM, with NO_3^-:NH_4^+ ratio of 3:1). Avocado shoots rooted at the rate of 100% after 4–8 weeks using a previously published protocol. The *in vitro* shoots and plantlets of avocado photosynthesized at a rate comparable to *in vivo* avocado plants, regardless of the presence of sugar in the medium. This indicated the viability of *in vitro*-grown avocado shoots and plantlets. This is the first report on the photosynthetic ability of *in vitro*-grown avocado shoots and plantlets.

Avocado protoplasts were isolated using the citrus protoplast isolation protocol (Appendix 4) from 8–14 day-old embryogenic suspension cultures that had been subcultured at 2-week intervals. Protoplast yields of ca. 1×10^6–3×10^7 have been routinely obtained depending on the genotype and morphology of the culture. Culture condition for growth, initiation and maturation of somatic embryos from protoplasts have been optimized. Under optimum conditions, protoplasts differentiated directly into proembryos and subsequently into somatic embryos. Alternatively microcallus formed prior to somatic embryo formation, and was dependent upon genotype and morphology of the source tissue for protoplast isolation. This is the first report of somatic embryo regeneration from avocado protoplasts.

Leaf mesophyll protoplasts have been isolated from expanding tender leaves from *in vitro*-grown seedlings of *Nectandra coriacea*, *Persea borbonia*, and *P. pachypoda* using protoplast isolation protocols developed for citrus leaves (Grosser & Chandler, 1987) with slight modifications. Protoplast yields varied with respect to leaf quality and can reach 3×10^7 protoplasts per gram of leaf tissue. This is the first report of leaf mesophyll protoplast isolation from these plants

Protoplasts from avocado embryogenic culture line 'T362' have been fused with mesophyll protoplasts of *Nectandra coriacea* using PEG-mediated chemical fusion (Grosser & Gmitter, 1990). Fusion frequencies reached ca. 13% when protoplasts of avocado and *Nectandra coriacea* were mixed in a ratio of 1:3–1:4. Large numbers of somatic embryos have been regenerated from several fusion experiments involving avocado and *Nectandra coriacea*; however, only one somatic embryo converted (one out of 600 somatic embryos) into a plantlet. The shoot was proliferated *in vitro* and was compared with plantlets derived from a 'T362' somatic embryo. RAPD analysis did not confirm that this plantlet was a somatic hybrid. Protoplasts have also been fused between avocado lines 'Thomas nucellus', 'Thomas zygotic' and 'Hass' that have lost their embryogenic potential and *P. pachypoda* and *P. borbonia*, although somatic embryos have

not been recovered. RAPD analysis of proembryonic masses that developed from fusion experiments demonstrated no DNA complementation of the parents. Protoplast fusion treatment itself improved the growth and differentiation of protoplasts.

Conclusion

A complete plant regeneration protocol for avocado through various interconnected tissue culture pathways based on somatic embryogenesis from either embryogenic cultures or protoplasts followed by shoot proliferation through axillary branching and rooting of the shoots has been improved or developed. These advances have several significant implications:

1. Application of somatic embryogenesis from nucellus explants of rootstock cultivars such as 'Thomas' or 'Duke 7' has potential for clonal propagation at a lower cost compared to the conventional 'etiolation' technique that is currently used.
2. The viability of protoplast fusion as a method for developing PRR-resistant rootstocks for avocado remains unproven. This is due to the limited number of fusion that have been performed. Higher numbers of fusions, more diverse fusion combinations, the use of embryogenic cultures that had not lost their embryogenic potential, the use of alternative protoplast sources from wild *Persea* species and electrofusion ought to be evaluated before a final conclusion regarding the viability of the fusion method for avocado improvement can be reached.
3. Avocado embryogenic cultures or embryogenic protoplasts could serve as target tissues of option for genetic transformation to generate genetic variability for breeding purposes. *Agrobacterium*-mediated transformation of 'Thomas zygotic' embryogenic culture with GUS and NPT II genes has been

confirmed (Cruz-Hernandez *et al.*, 1998). Single gene transformation of current rootstocks, e.g., 'Thomas', with antifungal genes or transformation of scion cultivars, e. g., 'Hass' with ripening genes for controlling fruit ripening would also be attractive.

The realization of all these potentials for avocado improvement, however, require the improvement of plant conversion or shoot development from the somatic embryos.

APPENDIX
AVOCADO TISSUE CULTURE MEDIA, PROTOPLASTS ISOLATION AND FUSION SOLUTIONS

Table A-1. Avocado tissue culture media.

COMPONENT	B5[S]	MSP[T]	SED[U]	SEMG[V]	SM[W]	ASP[X]	RI[Y]	RD[Z]
MS Major Salts				mg l^{-1}				
NH_4NO_3	0	1650	1650	1650	0	800	0	0
KNO_3	2527.5	1900	1900	1900	1900	2200	1900	1900
$(NH_4)_2SO_4$	134	0	0	0	0	0	0	0
$CaCl_2$ $2H_2O$	150	440	440	440	440	440	440	440
$MgSO_4$ $7H_2O$	246.5	370	370	370	370	370	370	370
KH_2PO_4	0	170	170	170	170	170	170	170
NaH_2PO_4	150	0	0	0	0	0	0	0
MS Minor Salts[1]				mg l^{-1}				
KI	0.830	0.830	0.830	0.830	0.830	0.830	0.830	0.830
H_3BO_3	6.200	6.200	6.200	6.200	6.200	6.200	6.200	6.200
$MnSO_4$ H_2O	16.900	16.900	16.900	16.900	16.900	16.900	16.900	16.900
$ZnSO_4$ $7H_2O$	8.600	8.600	8.600	8.600	8.600	8.600	8.600	8.600
Na_2MoO_4 $2H_2O$	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250
$CuSO_4$ $5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$CoCl_2$ $6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
FeNaEDTA	36.700	36.700	36.700	36.700	36.700	36.700	36.700	36.700
Organic Addenda				mg l^{-1}				
Thiamine HCl	4	4	4	4	4	4	4	4
myo-Inositol	100	100	100	100	100	100	100	100
Sucrose	30000	30000	30000	30000	30000	30000	30000	30000

Table A-1-continued

COMPONENT	B5[S]	MSP[T]	SED[U]	SEMG[V]	SM[W]	ASP[X]	RI[Y]	RD[Z]
				mg l^{-1} (μM)				
Plant Growth Regulator								
Picloram	0.1 (0.41)	0.1 (0.41)	0	0	0	0	0	0
IBA	0	0	0	0	0	0	25 (122.6)	0
BA	0	0	0	1 (4.44)	1 (4.44)	1 (4.44)	0	0
GA_3	0	0	0	1 (2.89)	0	0	0	0
Inert Support				g l^{-1}				
TC Agar	8	8	0	0	8	8	8	8
Gel-Gro	0	0	0	6–7	0	0	0	0
Activated Charcoal	0	0	0	0	0	0	0	1

[S] B5 (Gamborg et al., 1968) as induction medium for avocado embryogenic culture
[T] Maintenance medium. For MSP liquid medium, omit agar and filter sterilize medium with 0.2 μm pore opening
[U] Somatic Embryo Development medium. For liquid medium, omit Gel-Gro
[V] Somatic Embryo Maturation and Germination medium
[W] Shoot Multiplication medium (Witjaksono, 1991)
[X] Avocado Shoot Proliferation medium
[Y] Root Induction medium (modified from Pliego-Alfaro, 1988)
[Z] Root Development medium (modified from Pliego-Alfaro, 1988)

Table A-2. Avocado protoplast culture medium MS⁻8P.

Component	Amount of Stock (Ml)	Final Concentration in Medium
MS Major Salt Stock (devoid NH_4NO_3)	100	see Appendix 1
MS Minor Salt Stock	10	see Appendix 1
Thiamine-HCl Stock	10	1 mg l^{-1}
myo-Inositol	-	100 mg l^{-1}
8P Multivitamin Stock A	2	see Appendix 3
8P Multivitamin Stock B	1	see Appendix 3
8P Sugar and Sugar Alcohol Stock	10	see Appendix 3
8P Organic Acid Stock	20	see Appendix 3
Liquid Coconut Endosperm		0.2%
Malt Extract	-	1.000 g l^{-1}
Glutamine	-	3.100 g l^{-1}
Casein Hydrolysate	-	0.250 g l^{-1}
Sucrose	-	51.3 g l^{-1} (0.15 M)
Mannitol[z]	-	100.1 g l^{-1} (0.55 M)

Filter sterilize with 0.2 μm pore opening

This medium is a modification of BH3 medium (Grosser & Gmitter, 1990) in which the inorganic salts and vitamin are replaced with inorganic salts of MS without ammonium nitrate and 1 mg l^{-1} thiamine HCl. MS8P⁻ uses standard MS major salts and no glutamine is added to medium.

[z] Only mannitol concentration is altered for different medium osmolarity, while sucrose concentration in the medium stays the same. For 0.6 M MS⁻8P use 81.99 g l^{-1} mannitol, for 0.4 M MS⁻8P use 45.5 g l^{-1} mannitol, for 0.15 M MS⁻8P use no mannitol.

Table A-3. Stock solutions of 8P organic addenda (Kao & Michayluk,1975) as modified by Grosser and Gmitter (1990) and their final concentration in protoplast culture medium.

Component	Stock Solution (g/100 ml)	Final Concentration in the Medium (mg l^{-1})
Sugar and Sugar Alcohol (100X)		
Fructose	2.5	250
Ribose	2.5	250
Xylose	2.5	250
Mannose	2.5	250
Rhamnose	2.5	250
Cellobiose	2.5	250
Galactose	2.5	250
Mannitol	2.5	250
Organic Acid (50X)		
Sodium pyruvate	0.1	20
Citric acid	0.2	40
Malic acid	0.2	40
Fumaric acid	0.2	40
Multivitamin A		
Calcium pantothenate	0.05	1
Ascorbic acid	0.1	2
Choline chloride	0.05	1
p-Aminobenzoic acid	0.001	0.02
Folic acid	0.02	0.4
Riboflavin	0.01	0.2
Biotin	0.001	0.01
Multivitamin B		
Retinol (Vit. A)	0.001	0.01
Cholecalciferol (Vit. D_3)**	0.001	0.01
Cyanocobalamine (Vit.B_{12})	0.002	0.02

** insoluble in water, soluble in ethanol

Table A-4. Enzyme Solution for Protoplast Isolation.

Component	Stock in 100 ml	Amount in 40 ml Solution	Final Concentration
Mannitol	-	5.12 g	0.7 M
$CaCl_2$	14.4 g	1 ml stock	24.5 mM
NaH_2PO_4	0.44 g	1 ml stock	0.92 mM
M.E.S[1]	4.8 g	1 ml stock	6.25 mM
Cellulase Onozuka RS[x,y]	-	0.40 g	1% (w/v)
Macerase R10[x,z]	-	0.40 g	1% (w/v)
Pectolyase Y-23[x]	-	0.08 g	0.2% (w/v)

***Adjust pH to 5.6-6.0 with KOH
***Filter sterilize with 0.2 μm unit

From Grosser and Chandler (1987)
[x] Karlan Chemical, Santa Rosa, California
[y] Can be replaced with product from Kinki Yakult, Japan
[z] Can be replaced with Macerase® from Calbiochem®, La Jolla, California

Table A-5. CPW salts Stock Solutions and Their Final Concentration in the Solution.

Component	Amount in 100 ml Stock Solution (g)	Final Concentration ($mg\ l^{-1}$)
CPW Salts Stock 1[z]		
KH_2PO_4	0.272	27.2
KNO_3	1.00	100
$MgSO_4\ 7H_2O$	2.50	250
KI	0.0016	0.16
$CuSO_4\ 5H_2O$	0.000025	0.00025
CPW Salt Stock 2		
$CaCl_2\ 2H_2O$	1.5	150

From Frearson et al. (1973)
[z] 2.5 $mg.l^{-1}$ $Fe_2(SO_4)_3\ 6\ H_2O$ was omitted from original formulation.

Table A-6. Amount of CPW Stock Solution and Osmoticum per 100 ml Gradient Centrifugation Solution.

COMPONENT	CPW13MAN	CPW25SUC	CPW35SUC
CPW Stock 1	1 ml	1 ml	1 ml
CPW Stock 2	1 ml	1 ml	1ml
Sucrose	13 g	25 g	35 g
Mannitol			

pH were adjusted to 5.8 with KOH.
Solutions were filter sterilized with 0.2 µm pore opening.
Store in cold.

Table A-7. Protoplast Fusion Solution.

Component	Amount in 100 ml	Concentration
PEG Solution		
Polyethylene Glycol (1500 MW, Aldrich)	40 g	40% (w/v)
$CaCl_2$ $2H_2O$	0.97 g	66 mM
Glucose	5.41 g	0.3 M
***Adjust pH to 6.0 with KOH		
Solution A		
Glucose	7.2 g	0.4 M
$CaCl_2$ $2H_2O$	0.97 g	66 mM
Dimethylsulfoxide (DMSO)	10 ml	10% (v/v)
***Adjust pH to 6.0 with KOH		
Solution B		
Glycine	2.25 g	0.3 M
***Adjust pH to10.5 with KOH pellets		

From Menczel et al. (1981) as modified by Grosser and Gmitter (1990) and Grosser (1994).
All solutions were filter sterilized with millipore 0.2 µm unit.

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BIOGRAPHICAL SKETCH

Witjaksono was born on 8 October 1961 in Madiun, East Java Province, Indonesia, the son of the late Sunoto and his mother, Dian Tjahjani. He grew up in Madiun and attended schools in that city. After graduating from high school in 1980, he pursued his studies at Bogor Agricultural University (IPB), in Bogor, Indonesia, and earned a bachelor level degree, "Sarjana Pertanian," in agronomy in 1985.

During his final year at IPB, he received a scholarship and a commitment to work following the completion of his degree from the Indonesian Institute of Sciences (LIPI). After his graduation, he worked at the Center for Research and Development in Biology of LIPI. As a result of a competitive examination two years later, he received an overseas fellowship from the Indonesian government. With that fellowship he was admitted into the Department of Botany and Plant Sciences of the University of California at Riverside in order to study plant tissue culture techniques. He received his Master of Science degree in 1989. After working at LIPI for several years, he was again awarded an overseas fellowship to continue his graduate education. In 1992 he started his doctoral program in the Department of Horticultural Sciences, University of Florida. He spent two semesters in Gainesville taking classes and then began his research at the Tropical Research and Education Center, Homestead.

He is married to Dyah Kaniasari and blessed with two lovely sons, 5-year-old Lintang Adyuta Sutawika and 2-year-old Edgar Buwana Sutawika.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard E. Litz, Chair
Professor of Horticultural Science

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Jude W. Grosser
Professor of Horticultural Science

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December, 1997

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